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References

1. **Kanis JA, Melton III LJ, Christiansen C, Johnston CC, Khaltaev N** 1994 The diagnosis of osteoporosis. J Bone Miner Res 9:1137–1141
2. **Pocock NA, Eisman JA, Hopper JL, Yeates MG, Sambrook PN, Eberl S** 1987 Genetic determinations of bone mass in adults: a twin study. J Clin Invest 80:706–710
3. **Devoto M, Shimoya K, Caminis J, Ott J, Tenenhouse A, Whyte MP, Sereda L, Hall S, Considine E, Williams CJ, Tromp G, Kuivaniemi H, Ala-Kokko L, Prockop DJ, Spotila LD** 1998 First-stage autosomal genome screen in extended pedigrees suggests genes predisposing to low bone mineral density on chromosomes 1p, 2p and 4p. Eur J Hum Genet 6:151–157
4. **Johnson ML, Gong G, Kimberling W, Recker SM, Kimmel DB, Recker RB** 1997 Linkage of a gene causing high bone mass to human chromosome 11 (11q12–13). Am J Hum Genet 60:1309–1311
5. **Uitterlinden AG, Burger H, Huang Q, Yue F, McGuigan FEA, Grant SFA, Hofman A, van Leeuwen JPTM, Pols HAP, Ralston SH** 1998 Relation of alleles of the collagen type Iα1 gene to bone density and the risk of osteoporotic fractures in postmenopausal women. N Engl J Med 338:1016–1021
6. **Yamada Y, Ando F, Niino N, Shimokata H** 2001 Transforming growth factor-β1 gene polymorphism and bone mineral density. JAMA 285:167–168
7. **Manolagas SC, Jilka RL** 1995 Bone marrow, cytokines, and bone remodeling: emerging insights into the pathophysiology of osteoporosis. N Engl J Med 332:305–311
8. **Ota N, Hunt SC, Nakajima T, Suzuki T, Hosoi T, Orimo H, Shirai Y, Emi M** 1999 Linkage of interleukin 6 locus to human osteopenia by sibling pair analysis. Hum Genet 105:253–257
9. **Ota N, Nakajima T, Nakazawa I, Suzuki T, Hosoi T, Orimo H, Inoue S, Shirai Y, Emi M** 2001 A nucleotide variant in the promoter region of the interleukin-6 gene associated with decreased bone mineral density. J Hum Genet 46:267–272
10. **Murray RE, McGuigan F, Grant SFA, Reid DM, Ralston SH** 1997 Polymorphisms of the interleukin-6 gene are associated with bone mineral density. Bone 21:89–92
11. **Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Karsenty G** 1996 Increased bone formation in osteocalcin-deficient mice. Nature 382:448–452
12. **Dohi Y, Iki M, Ohgushi H, Gojo S, Tabata S, Kajita E, Nishino H, Yonemasu K** 1998 A novel polymorphism in the promoter region for the human osteocalcin gene: the possibility of a correlation with bone mineral density in postmenopausal Japanese women. J Bone Miner Res 13:1633–1639
13. **Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA** 1994 Prediction of bone density from vitamin D receptor alleles. Nature 367:284–287
14. **Gross C, Eccleshall TR, Malloy PJ, Villa ML, Marcus R, Feldman D** 1996 The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women. J Bone Miner Res 11:1850–1855
15. **Arai H, Miyamoto K, Taketani Y, Yamamoto H, Iemori Y, Morita K, Tonai T, Nishisho T, Mori S, Takeda E** 1997 A vitamin D receptor gene polymorphism in the translation initiation codon: effect on protein activity and relation to bone mineral density in Japanese women. J Bone Miner Res 12:915–921
16. **Eccleshall TR, Garnero P, Gross C, Delmas PD, Feldman D** 1998 Lack of correlation between start codon polymorphism of the vitamin D receptor gene and bone mineral density in premenopausal French women: the OFELY study. J Bone Miner Res 13:31–35
17. **Ferrari S, Manen D, Bonjour J-P, Slosman D, Rizzoli R** 1999 Bone mineral mass and calcium and phosphate metabolism in young men: relationships with vitamin D receptor allelic polymorphisms. J Clin Endocrinol Metab 84:2043–2048
18. **Kanan RM, Varanasi SS, Francis RM, Parker L, Datta HK** 2000 Vitamin D receptor gene start codon polymorphism (*Fok* I) and bone mineral density in healthy male subjects. Clin Endocrinol (Oxf) 53:93–98
19. **Ames SK, Ellis KJ, Gunn SK, Copeland KC, Abrams SA** 1999 Vitamin D receptor gene *Fok* I polymorphism predicts calcium absorption and bone mineral density in children. J Bone Miner Res 14:740–746
20. **Shimokata H, Ando F, Niino N** 2000 A new comprehensive study on aging—the National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS–LSA). J Epidemiol 10:S1–S9
21. **Gross C, Krishnan AV, Malloy PJ, Eccleshall TR, Zhao X-Y, Feldman D** 1998 The vitamin D receptor gene start codon polymorphism: a functional analysis of *Fok* I variants. J Bone Miner Res 13:1691–1699

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ORIGINAL ARTICLE

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Association of polymorphisms of paraoxonase 1 and 2 genes, alone or in combination, with bone mineral density in community-dwelling Japanese

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Abstract Oxidative stress may affect cellular functions in various pathological conditions, including osteoporosis. Paraoxonase 1 confers antioxidant properties on high-density lipoprotein, with which it is associated, by reducing the accumulation of lipid peroxidation products. We have now examined whether the 584A → G (Gln192Arg) and 172T → A (Leu55Met) polymorphisms of the paraoxonase 1 gene and the 959G → C (Cys311Ser) polymorphism of the paraoxonase 2 gene are associated with bone mineral density (BMD) in community-dwelling Japanese (1,087–1,094 women and 1,112–1,125 men). The subjects were aged 40 –79 years and were randomly recruited to a population-based prospective cohort study of aging and age-related diseases. BMD for the lumbar spine and right femoral neck was measured by dual-energy X-ray absorptiometry. Genotypes were determined with a fluorescence- or colorimetry-based allele-specific DNA primer-probe assay system. The 584A → G and 172T → A polymorphisms of the paraoxonase 1 gene and the 959G → C polymorphism of the paraoxonase 2 gene were associated with BMD for the lumbar spine or femoral neck in postmenopausal women, with the 584*GG*, 172*TT*, and 959*CC* genotypes representing risk factors for reduced bone mass. None of these three polymorphisms was associated with BMD in premenopausal women or in men. Our results suggest that the paraoxonase 1 and 2 genes are candidate loci for reduced bone mass in postmenopausal Japanese women.

Keywords Bone mineral density · Genetics · Osteoporosis · Paraoxonase · Polymorphism

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Introduction

Osteoporosis is characterized by a reduction in bone mineral density (BMD) and a deterioration in the microarchitecture of bone, both of which result in an increased susceptibility to fractures (Kanis et al. 1994). Although several environmental factors, such as diet and physical exercise, influence BMD, a genetic contribution to the etiology of osteoporosis has been recognized (Pocock et al. 1987). Genetic linkage analyses (Devoto et al. 1998; Johnson et al. 1997; Morrison et al. 1994) and candidate gene association studies (Morrison et al. 1994; Uitterlinden et al. 1998; Yamada et al. 2001) have thus implicated several loci and candidate genes in the regulation of bone mass and the pathogenesis of osteoporotic fractures. The genes that contribute to genetic susceptibility to osteoporosis, however, remain to be identified definitively.

Oxidative stress may affect cellular functions in various pathological conditions, including osteoporosis (Basu et al. 2001; Garrett et al. 1990). Recent evidence has suggested that lipid oxidation contributes to the development of osteoporosis (Parhami et al. 2000). In vitro studies indicate that lipid oxidation products promote osteoblastic differentiation of vascular cells and inhibit such differentiation of bone cells (Parhami et al. 1997). Oxidation products of low-density lipoprotein (LDL) also promote osteoporotic loss of bone by inducing progenitor marrow stromal cells to undergo adipogenic rather than osteogenic differentiation (Parhami et al. 1999).

Paraoxonase 1 (PON1) is a calcium-dependent esterase that is closely associated with high-density

lipoprotein (HDL)-containing apolipoprotein A-I and is thought to confer antioxidant properties on HDL by preventing the accumulation of lipid peroxidation products (Mackness et al. 1991). This property of PON1 accounts for its ability to protect against atherosclerosis. In addition to the PON1 gene (*PON1*), related genes designated *PON2* and *PON3* have been identified in the human genome and are linked with *PON1* on chromosome 7q21-q22 (Primo-Parmo et al. 1996). Two single nucleotide polymorphisms (SNPs), 584A → G (Gln192 Arg; GenBank accession no. M63012) and 172T → A (Leu55Met; accession no. M63012), of *PON1* and a 959G → C (Cys311Ser; accession no. L48513) SNP of *PON2* have been associated with coronary artery disease (Ruiz et al. 1995; Serrato and Marian 1995; Garin et al. 1997; Sanghera et al. 1998). Although PON1 catalyzes the reduction of oxidized LDL and thereby may affect bone remodeling, the possible relations of these SNPs to BMD have not been determined.

We have now examined whether the 584A → G (Gln192Arg) and 172T → A (Leu55Met) SNPs of *PON1* and the 959G → C (Cys311Ser) SNP of *PON2* are associated with BMD in women or men in a large-scale, population-based study.

Subjects and methods

Study population

The National Institute for Longevity Sciences—Longitudinal Study of Aging (NILS-LSA) is a population-based prospective cohort study of aging and age-related diseases (Shimokata et al. 2000). The subjects of the NILS-LSA are stratified by both age and gender and are randomly selected from resident registrations in the city of Obu and town of Higashiura in central Japan. The lifestyle of residents of this area is typical of that of individuals in most regions of Japan. The numbers of men and women recruited are similar, and the baseline age is 40–79 years, with similar numbers of participants in each decade (40s, 50s, 60s, 70s). The subjects will be followed up every 2 years.

All participants are subjected at a special center to a detailed examination, which includes not only medical evaluation but also assessment of exercise physiology, body composition, nutrition, and psychology. Individuals with disorders known to cause abnormalities of bone metabolism, including diabetes mellitus, renal diseases, rheumatoid arthritis, as well as thyroid, parathyroid, and other endocrinologic diseases, were excluded from the study. Women who had taken drugs such as estrogen, progesterone, glucocorticoids, and bisphosphonates were also excluded.

We examined the possible association of BMD with the 584A → G (Gln192Arg) SNP of *PON1* in 2,199 participants (1,087 women, 1,112 men), with the 172T → A (Leu55Met) SNP of *PON1* in 2,210 participants (1,092 women, 1,118 men), and with the 959G → C (Cys311Ser) SNP of *PON2* in 2,219 participants (1,094 women, 1,125 men). The study protocol was approved by the Committee on Ethics of Human Research of National Chubu Hospital and the NILS, and written informed consent was obtained from each subject.

Measurement of BMD

BMD for the lumbar spine (L2–L4) and right femoral neck was measured by dual-energy X-ray absorptiometry (DEXA) (QDR 4500; Hologic, Bedford, MA, USA). The coefficients of variance of the DEXA instrument were 0.9% for L2–L4 and 1.3% for the femoral neck.

Determination of genotypes

Genotypes were determined with a fluorescence- or colorimetry-based allele-specific DNA primer-probe assay system (Toyobo Gene Analysis, Tsuruga, Japan).

For determination of genotype of the 584A → G (Gln192Arg) SNP, the polymorphic region of *PON1* was amplified by the polymerase chain reaction (PCR) with a sense primer labeled at the 5' end with biotin (5'-GAATGATATTGTTGCTGTGGGACC-3') and allele-specific antisense primers (5'-AACCCAAATACATCTCCC-AGGAXTG-3' or 5'-ACCCAAATACATCTCCCAGGAXCG-3').

For determination of 172T → A (Leu55Met) genotype, the polymorphic region of *PON1* was similarly amplified with a sense primer (5'-TCTGGCAGAAACTGGCTCTGAA-3') and an antisense primer labeled at the 5' end with biotin (5'-GCTA-ATGAAAGCCAGTCCATTA-3').

For determination of 959G → C (Cys311Ser) genotype, the polymorphic region of *PON2* was amplified by PCR with allele-specific sense primers labeled at the 5' end with either fluorescein isothiocyanate (5'- CGCATCCAGAACATTCTAXGT-3') or Texas red (5'- CCGCATCCAGAACATTCTAXCT-3') and with an antisense primer labeled at the 5' end with biotin (5'-GGCA-TAAACTGTAGTCACTGTAGGC-3').

The reaction mixtures (25 μl) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 1.4 mmol/l $MgSO_4$ (584A → G and 172T → A genotypes) or 1.8 mmol/l $MgCl_2$ (959G → C genotype), and 1 U of DNA polymerase (rTaq or KODplus; Toyobo, Osaka, Japan) in the respective DNA polymerase buffer. The amplification protocol comprised initial denaturation at 95°C for 5 min; 35 cycles (584A → G and 959G → C genotypes) or 40 cycles (172T → A genotype) of denaturation at 95°C for 30 s, annealing at 63°C (584A → G genotype), 55°C (172T → A genotype), or 60°C (959G → C genotype) for 30 s, and extension at 68°C for 30 s; and a final extension at 68°C for 2 min.

For determination of 584A → G and 172T → A genotypes, amplified DNA was denatured with 0.3 mol/l NaOH and then subjected to hybridization at 37°C for 30 min in hybridization buffer containing 35% (584A → G genotype) or 32.5% (172T → A genotype) formamide with allele-specific capture probes (5'-ACATCTCCCAGGAXTGTAAGTAG-3' or 5'-ACA-TCTCCCAGGAXCGTAAGTAG-3' for 584A → G genotype, and 5'-GAAGACTTGGAGATACTGCC-3' or 5'-GAAGACATG-GAGATACTGCC-3' for 172T → A genotype) fixed to the bottom of the wells of a 96-well plate. After thorough washing of the wells, alkaline phosphatase-conjugated streptavidin was added to each, and the plate was incubated at 37°C for 15 min with agitation. The wells were again washed, and after the addition of a solution containing 0.8 mmol/l 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (monosodium salt) and 0.4 mmol/l 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, absorbance at 450 nm was measured.

For determination of 959G → C genotype, amplified DNA was incubated in a solution containing streptavidin-conjugated magnetic beads in the wells of a 96-well plate at room temperature. The plate was placed on a magnetic stand, and the supernatants were then collected from each well, transferred to the wells of a 96-well plate containing 0.01 mol/l NaOH, and measured for fluorescence with a microplate reader (Fluoroscan Ascent; Dainippon Pharmaceutical, Osaka, Japan) at excitation and emission wavelengths of 485 and 538 nm, respectively, for fluorescein isothiocyanate and of 584 and 612 nm, respectively, for Texas red.

To confirm the accuracy of genotyping by this method, we selected 50 DNA samples at random and subjected them to PCR and restriction fragment-length polymorphism analysis or to direct DNA sequencing of PCR products. In each instance, the genotype determined by the allele-specific DNA primer-probe assay system was identical to that determined by the confirmatory methods.

Assay for serum activity of PON1

Venous blood was collected from subjects after an overnight fast. Blood samples were centrifuged at 1600×*g* for 15 min at 4°C, and serum was separated and stored at −80°C until assayed. The serum activity of PON1 was measured as previously described (Mackness et al. 1997). The intra- and interassay coefficients of variance were <2.6 and <2.3%, respectively.

Statistical analysis

Quantitative data were compared among three groups by one-way analysis of variance and the Tukey-Kramer post hoc test, and between two groups by the unpaired Student's *t* test. BMD values were analyzed with or without adjustment for age by the least squares method in a general linear model (SAS, SAS Institute, Cary, NC, USA). Allele frequencies were estimated by the gene-counting method, and the chi-square test was used to identify significant departure from Hardy-Weinberg equilibrium. Haplotype analysis was performed with SAS/Genetics (SAS Institute). Correlation between the serum activity of PON1 and BMD was determined by simple regression analysis. Unless indicated otherwise, a *P* value of < 0.05 was considered statistically significant.

Results

The distribution of combined genotypes of three SNPs is shown in Table 1. Linkage disequilibrium was apparent between the 584A → G (Gln192Arg) and 172T → A (Leu55Met) SNPs of *PON1* [*D'*, pairwise linkage disequilibrium coefficient (*D*/*D*max), 0.4254; *r*, standardized linkage disequilibrium coefficient, 0.1673; *P* <0.0001], between the *PON1* 584A → G and *PON2* 959G → C (Cys311Ser) SNPs (*D'*, 0.2112; *r*, 0.1470; *P* <0.0001), and between the *PON1* 172A → T and *PON2* 959G → C SNPs (*D'*, 0.2491; *r*, 0.1421; *P* <0.0001).

Characteristics of study subjects according to genotypes for the 584A → G SNP or 172T → A SNP of *PON1* or for the 959G → C SNP of *PON2* are shown in Table 2. The distributions of genotypes were in Hardy-Weinberg equilibrium for premenopausal or postmenopausal women or for men in three SNPs. There was no difference in age or body mass index for premenopausal or postmenopausal women or for men among genotypes of each SNP.

The relation of the 584A → G SNP of *PON1* to BMD was examined (Table 3). To examine the possible influence of menopause on the relation between genotype and BMD, we analyzed BMD and other characteristics for premenopausal and postmenopausal women independently. Given the multiple comparisons of genotypes with BMD, we considered a *P* value of < 0.01 to be significant for such associations. For premenopausal women, BMD was not associated with 584A → G genotype with or without adjustment for age. In contrast, for postmenopausal women, BMD for the femoral neck was significantly lower in those with the *GG* genotype than in those with the *GA* or *AA* genotype. After adjustment for age, BMD for the femoral neck was also significantly lower in postmenopausal women with the *GG* genotype than in those with the *GA* or *AA* genotype or the *GA* genotype. The difference in adjusted BMD between the *GG* genotype and the *GA* or *AA* genotype (expressed as a percentage of the corresponding larger value) in postmenopausal women was

Table 1 Distribution of combined genotypes of polymorphisms of *PON1* and *PON2* among study subjects (n=2,196). Data are numbers of subjects (%)

PON1 584A → G	*PON1* 172T → A			*PON2* 959G → C
	TT	*TA*	*AA*	
GG	811 (36.93)	0 (0)	0 (0)	*CC*
	141 (6.42)	0 (0)	0 (0)	*CG*
	13 (0.59)	0 (0)	0 (0)	*GG*
GA	470 (21.40)	59 (2.69)	0 (0)	*CC*
	292 (13.30)	133 (6.06)	1 (0.05)	*CG*
	26 (1.18)	15 (0.68)	1 (0.05)	*GG*
AA	64 (2.91)	18 (0.82)	2 (0.09)	*CC*
	57 (2.60)	36 (1.64)	8 (0.36)	*CG*
	19 (0.87)	24 (1.09)	6 (0.27)	*GG*

Table 2 Characteristics of study subjects according to the 584A → G (Gln192Arg) or 172T → A (Leu55Met) genotype of *PON1* or the 959G → C (Cys311Ser) genotype of *PON2*. Data are means ± SE. *BMI* body mass index

Characteristic	584A → G (Gln192Arg)			172T → A (Leu55Met)			959G → C (Cys311Ser)		
	GG	*GA*	*AA*	*TT*	*TA*	*AA*	*CC*	*CG*	*GG*
Premenopausal women									
Number (%)	107 (38.6)	136 (49.1)	34 (12.3)	239 (86.0)	35 (12.6)	4 (1.4)	173 (62.2)	92 (33.1)	13 (4.7)
Age (years)	46.1±0.4	46.2±0.4	46.6±0.8	46.0±0.3	47.1±0.8	48.3±2.3	46.2±0.4	46.2±0.5	45.7±1.3
BMI (kg/m^2)	22.9±0.3	22.7±0.3	22.7±0.6	22.8±0.2	22.5±0.5	22.2±1.6	22.9±0.2	22.6±0.3	22.8±0.9
Postmenopausal women									
Number (%)	369 (45.6)	353 (43.6)	88 (10.9)	702 (86.2)	107 (13.1)	5 (0.6)	551 (67.5)	227 (27.8)	38 (4.7)
Age (years)	64.1±0.4	63.8±0.5	64.1±0.9	64.1±0.3	63.4±0.8	63.4±3.8	64.1±0.4	63.5±0.6	63.9±1.4
BMI (kg/m^2)	22.8±0.2	23.0±0.2	23.5±0.4	23.0±0.1	22.7±0.3	23.9±1.5	22.9±0.1	23.2±0.2	23.3±0.5
Men									
Number (%)	493 (44.3)	504 (45.3)	115 (10.3)	965 (86.3)	144 (12.9)	9 (0.8)	720 (64.0)	351 (31.2)	54 (4.8)
Age (years)	59.2±0.5	59.2±0.5	58.7±1.0	59.0±0.4	60.0±0.9	59.4±3.6	59.1±0.4	59.2±0.6	59.2±1.5
BMI (kg/m^2)	22.9±0.2	23.1±0.2	22.6±0.3	23.0±0.1	22.8±0.2	24.0±1.2	23.1±0.1	22.7±0.2	23.0±0.4

3.1% for the femoral neck. For men, BMD for the lumbar spine or femoral neck did not differ among 584A → G genotypes with or without adjustment for age (data not shown).

The relation between the 172T → A SNP of *PON1* and BMD is shown in Table 4. For premenopausal women, BMD was not associated with 172T → A genotype with or without adjustment for age. For postmenopausal women, BMD for the lumbar spine or femoral neck was significantly lower in those with the *TT* genotype than in those with the *TA* or *AA* genotype or the *TA* genotype, with or without adjustment for age. The differences in adjusted BMD between the *TT* genotype and the *TA* or *AA* genotype in postmenopausal women were 5.3% for the lumbar spine and 4.0% for the femoral neck. For men, BMD did not differ among 172T → A genotypes with or without adjustment for age (data not shown).

The relation of the 959G → C SNP of *PON2* with BMD is shown in Table 5. BMD did not differ among 959G → C genotypes for premenopausal women with or without adjustment for age. For postmenopausal women, BMD for the femoral neck was significantly lower in those with the *CC* genotype than in those with the *CG* or *GG* genotype with or without adjustment for age. The difference in adjusted BMD between the *CC* genotype and the *CG* or *GG* genotype in postmenopausal women was 2.9% for the femoral neck. BMD did not differ significantly among 959G → C genotypes in men with or without adjustment for age (data not shown).

We examined the relation of haplotypes of the 584A → G, 172T → A, and 959G → C SNPs to BMD (Table 6). Given the small number of subjects with the *GAC* (one woman and no men) and *GAG* (no women and one man) haplotypes, these groups were excluded from the analysis. For postmenopausal women, BMD

Table 3 Bone mineral density (BMD) of women ($n = 1{,}087$) according to the 584A → G (Gln192Arg) genotype of *PON1*. Data are means ± SE

Characteristic	Premenopausal ($n = 277$)				Postmenopausal ($n = 810$)			
	GG	*GA*	*AA*	*GA + AA*	*GG*	*GA*	*AA*	*GA + AA*
L2–L4	1.035 ± 0.012	1.026 ± 0.011	0.988 ± 0.021	1.018 ± 0.010	0.797 ± 0.008†	0.816 ± 0.008	0.830 ± 0.016	0.819 ± 0.007
Adjusted L2–L4[a]	1.034 ± 0.012	1.026 ± 0.010	0.990 ± 0.021	1.019 ± 0.009	0.798 ± 0.007†	0.814 ± 0.007	0.831 ± 0.015	0.818 ± 0.007
Femoral neck	0.777 ± 0.010	0.776 ± 0.008	0.745 ± 0.017	0.770 ± 0.008	0.632 ± 0.005‡‖	0.655 ± 0.006	0.649 ± 0.011	0.654 ± 0.005
Adjusted femoral neck[a]	0.776 ± 0.009	0.776 ± 0.008	0.747 ± 0.016	0.770 ± 0.007	0.633 ± 0.005§††	0.654 ± 0.005	0.650 ± 0.009	0.653 ± 0.004

[a]BMD with adjustment for age
†$P < 0.05$
‡$P < 0.005$
§$P < 0.001$ versus *GA + AA*
‖$P < 0.01$
††$P < 0.005$ versus *GA*

Table 4 Bone mineral density (BMD) of women ($n = 1{,}092$) according to the 172T → A (Leu55Met) genotype of *PON1*. Data are means ± SE

Characteristic	Premenopausal ($n = 278$)				Postmenopausal ($n = 814$)			
	TT	*TA*	*AA*	*TA + AA*	*TT*	*TA*	*AA*	*TA + AA*
L2–L4	1.030 ± 0.008	0.996 ± 0.021	0.940 ± 0.062	0.990 ± 0.020	0.803 ± 0.006†§	0.852 ± 0.015	0.849 ± 0.068	0.852 ± 0.014
Adjusted L2–L4[a]	1.029 ± 0.008	1.002 ± 0.020	0.954 ± 0.060	0.997 ± 0.019	0.803 ± 0.005‡§	0.848 ± 0.013	0.845 ± 0.061	0.848 ± 0.013
Femoral neck	0.775 ± 0.006	0.754 ± 0.017	0.709 ± 0.050	0.749 ± 0.016	0.639 ± 0.004†‖	0.672 ± 0.010	0.628 ± 0.047	0.670 ± 0.010
Adjusted femoral neck[a]	0.774 ± 0.006	0.758 ± 0.016	0.720 ± 0.048	0.755 ± 0.015	0.640 ± 0.003†§	0.669 ± 0.009	0.625 ± 0.039	0.667 ± 0.008

[a]BMD with adjustment for age
†$P < 0.005$
‡$P < 0.001$ versus *TA + AA*
§$P < 0.005$
‖$P < 0.01$ versus *TA*

Table 5 Bone mineral density (BMD) of women ($n = 1{,}094$) according to the 959G → C (Cys311Ser) genotype of *PON2*. Data are means ± SE

Characteristic	Premenopausal ($n = 278$)				Postmenopausal ($n = 816$)			
	CC	*CG*	*GG*	*CG + GG*	*CC*	*CG*	*GG*	*CG + GG*
L2–L4	1.028 ± 0.009	1.024 ± 0.013	0.983 ± 0.034	1.019 ± 0.012	0.803 ± 0.006	0.825 ± 0.010	0.814 ± 0.025	0.823 ± 0.009
Adjusted L2–L4[a]	1.028 ± 0.009	1.024 ± 0.013	0.979 ± 0.033	1.019 ± 0.012	0.804 ± 0.006	0.822 ± 0.009	0.814 ± 0.022	0.820 ± 0.008
Femoral neck	0.769 ± 0.008	0.778 ± 0.010	0.756 ± 0.028	0.775 ± 0.010	0.637 ± 0.004†‡	0.660 ± 0.007	0.655 ± 0.017	0.659 ± 0.006
Adjusted femoral neck[a]	0.770 ± 0.007	0.778 ± 0.010	0.753 ± 0.027	0.775 ± 0.009	0.638 ± 0.004†‡	0.657 ± 0.006	0.655 ± 0.014	0.657 ± 0.005

[a]BMD with adjustment for age
†$P < 0.005$ versus *CG + GG*
‡$P < 0.05$ versus *CG*

Table 6 Bone mineral density (BMD) of premenopausal or postmenopausal women or of men according to haplotypes of three polymorphisms. Data are means ± SE

BMD	Haplotype [584A → G (*PON1*), 172T → A (*PON1*), 959G → C (*PON2*)]					
	GTC	*GTG*	*ATC*	*ATG*	*AAC*	*AAG*
Premenopausal women						
No. of chromosomes (%)	314 (57.40)	31 (5.67)	106 (19.38)	55 (10.05)	9 (1.65)	32 (5.85)
L2–L4	1.034 ± 0.007	1.012 ± 0.022	1.014 ± 0.012	1.033 ± 0.017	0.999 ± 0.041	0.986 ± 0.022
Femoral neck	0.776 ± 0.006	0.783 ± 0.018	0.766 ± 0.010	0.781 ± 0.013	0.734 ± 0.033	0.751 ± 0.018
Postmenopausal women						
No. of chromosomes (%)	1016 (63.18)	66 (4.10)	259 (16.11)	150 (9.33)	33 (2.05)	84 (5.22)
L2–L4	0.802 ± 0.005	0.832 ± 0.019	0.817 ± 0.009	0.802 ± 0.012	0.869 ± 0.026	0.845 ± 0.017
Femoral neck	0.638 ± 0.003*	0.662 ± 0.013	0.644 ± 0.007	0.658 ± 0.009	0.700 ± 0.018	0.656 ± 0.011
Men						
No. of chromosomes (%)	1366 (62.03)	110 (5.00)	339 (15.40)	227 (10.31)	46 (2.09)	113 (5.13)
L2–L4	0.979 ± 0.004	0.970 ± 0.015	0.989 ± 0.009	0.988 ± 0.011	0.993 ± 0.024	0.984 ± 0.015
Femoral neck	0.750 ± 0.003	0.747 ± 0.011	0.757 ± 0.006	0.756 ± 0.008	0.765 ± 0.017	0.751 ± 0.011

*$P < 0.01$ versus *AAC*

Table 7 Serum activity (nmol min^{-1} ml^{-1}) of PON1 in study subjects according to the 584A → G (Gln192Arg) and 172T → A (Leu55Met) genotypes of *PON1* and the 959G → C (Cys311Ser) genotype of *PON2*. Data are means ± SE

	Total subjects	Women	Men
584A → G (Gln192Arg) genotype			
GG	503.7 ± 3.0 ($n = 969$)	516.5 ± 4.1 ($n = 476$)	491.2 ± 4.2 ($n = 493$)
GA	325.6 ± 2.9 ($n = 993$)*	333.5 ± 4.0 ($n = 489$)*	317.9 ± 4.1 ($n = 504$)*
AA	139.3 ± 6.1 ($n = 237$)*†	145.7 ± 8.3 ($n = 122$)*†	132.2 ± 8.9 ($n = 115$)*†
172T → A (Leu55Met) genotype			
TT	407.2 ± 3.3 ($n = 1906$)	413.9 ± 4.7 ($n = 941$)	400.5 ± 4.5 ($n = 965$)
TA	256.5 ± 8.4 ($n = 286$)‡	268.7 ± 12.2 ($n = 142$)‡	244.5 ± 11.5 ($n = 144$)‡
AA	101.9 ± 32.9 ($n = 18$)‡§	111.5 ± 49.1 ($n = 9$)‡‖	93.3 ± 43.9 ($n = 9$)‡‖
959G → C (Cys311Ser) genotype			
CC	408.9 ± 3.9 ($n = 1444$)	415.6 ± 5.6 ($n = 724$)	402.0 ± 5.5 ($n = 720$)
CG	349.1 ± 5.8 ($n = 670$)††	361.7 ± 8.4 ($n = 319$)††	337.4 ± 7.9 ($n = 351$)††
GG	285.2 ± 15.1 ($n = 105$)††#	267.3 ± 21.9 ($n = 51$)††#	302.4 ± 20.7 ($n = 54$)††

*$P = 0.0001$ versus *GG*
†$P = 0.0001$ versus *GA*
‡$P = 0.0001$ versus *TT*
§$P = 0.0001$ versus *TA*
‖$P < 0.005$ versus *TA*
††$P = 0.0001$ versus *CC*
#$P < 0.0005$ versus *CG*

for the femoral neck was significantly lower in those with the *GTC* haplotype than in those with the *AAC* haplotype. For premenopausal women or for men, BMD did not differ among haplotypes.

We examined the relation of three SNPs with the serum activity of PON1 (Table 7). There was a significant association between the serum activity of PON1 and 584A → G, 172T → A, and 959G → C genotypes both for women and for men. With regard to the 584A → G genotype, the activity of PON1 was higher in individuals with the *GG* genotype than in those with the *GA* genotype or those with the *AA* genotype; the activity was also higher in those with the *GA* genotype than in those with the *AA* genotype. For the 172T → A genotype, the activity of PON1 was higher in individuals with the *TT* genotype than in those with the *TA* or those with the *AA* genotypes; the activity was also higher in those with the *TA* genotype than in those with the *AA* genotype. With respect to the 959G → C genotype, the serum activity of PON1 was higher in individuals with the *CC* genotype than in those with the *CG* or those with the *GG* genotypes; for total subjects and for women, the activity was also higher in those with the *CG* genotype than in those with the *GG* genotype.

Finally, we examined the correlation between the serum activity of PON1 and BMD (Fig. 1). There was no significant relation of the serum activity of PON1 with BMD for the lumbar spine ($r = 0.010$, $P = 0.844$) or for the femoral neck ($r = 0.014$, $P = 0.683$) in postmenopausal women.

Discussion

We have shown that the 584A → G (Gln192Arg) and 172T → A (Leu55Met) SNPs of *PON1* and the 959G → C (Cys311Ser) SNP of *PON2* are associated with BMD for the lumbar spine or femoral neck in postmenopausal Japanese women, and that the 584*GG*, 172*TT*, and 959*CC* genotypes represent risk factors for

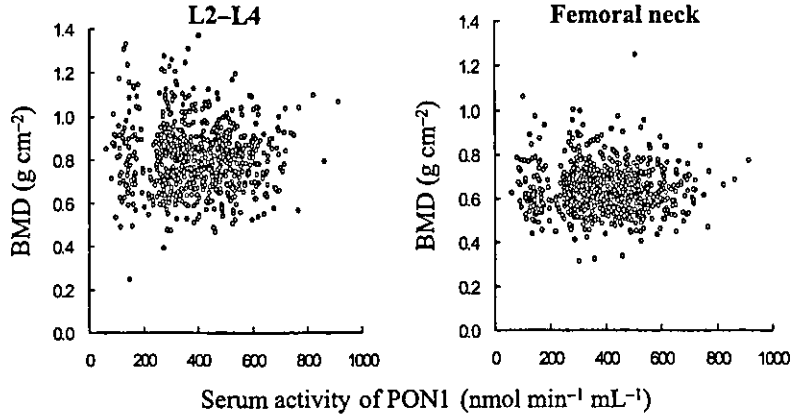

Fig. 1 Correlation of serum activity of PON1 with bone mineral density (BMD) for the lumbar spine (L2–L4) (*left panel*) or femoral neck (*right panel*) in postmenopausal women ($n = 816$)

reduced bone mass. Haplotype analysis revealed that the *GTC* haplotype exhibited the lowest BMD and the *AAC* haplotype the highest BMD in postmenopausal women. There was no significant association of polymorphisms of *PON1* or *PON2* with BMD in premenopausal women or in men.

The three SNPs examined in the present study have been previously associated with the activity of PON1 in serum or plasma. We measured the serum activity of PON1 in the present study population to confirm the effects of three SNPs on the activity. Humbert et al. (1993) determined that the 584*G* (192Arg) allele is associated with a higher activity of PON1 in plasma than is the 584*A* (192Gln) allele. Garin et al. (1997) showed that the plasma concentrations and activities of PON1 decreased according to the rank order of 172T → A (Leu55Met) genotypes *TT* > *TA* > *AA*. Our present results are consistent with these previous observations (Garin et al. 1997; Humbert et al. 1993).

Mackness et al. (2000) showed that the 959G → C (Cys311Ser) SNP of *PON2* also affects the serum activity of PON1; among individuals with type 2 diabetes, PON1 activity was highest in those with the 959*GG* genotype. In contrast, our results demonstrate that the *GG* genotype is associated with the lowest PON1 activity and that the *CC* genotype exhibits the highest activity for both women and men.

In the present study, the 584*GG*, 172*TT*, and 959*CC* genotypes, which exhibited the highest serum activity of PON1, were associated with reduced BMD in postmenopausal women. A high serum activity of PON1 may result in a reduced concentration of lipid peroxidation products and might therefore be expected to prevent bone loss (Basu et al. 2001; Garrett et al. 1990; Parhami et al. 1997; Parhami et al. 1999; Parhami et al. 2000). The association between genotypes and BMD in our study is thus opposite to that anticipated from such a mechanism. The molecular mechanisms that underlie the association of SNPs of *PON1* and *PON2* with BMD thus remain unclear. It is possible that the SNPs examined in our study are in linkage disequilibrium with polymorphisms of other nearby genes that are determinants of BMD. Indeed, the interleukin-6 gene (7q21) and calcitonin receptor gene (7q21.3), both of which have been associated with BMD (Ota et al. 1999; Ota et al. 2001; Taboulet et al. 1998), are located close to *PON1* and *PON2* (7q21-q22). However, our present results suggest that *PON1* and *PON2* are candidate loci for reduced bone mass in postmenopausal Japanese women.

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References

Basu S, Michaelsson K, Olofsson H, Johansson S, Melhus H (2001) Association between oxidative stress and bone mineral density. Biochem Biophys Res Commun 288:275–279

Devoto M, Shimoya K, Caminis J, Ott J, Tenenhouse A, Whyte MP, Sereda L, Hall S, Considine E, Williams CJ, Tromp G, Kuivaniemi H, Ala-Kokko L, Prockop DJ, Spotila LD (1998) First-stage autosomal genome screen in extended pedigrees suggests genes predisposing to low bone mineral density on chromosomes 1p, 2p and 4p. Eur J Hum Genet 6:151–157

Garin M-CB, James RW, Dussoix P, Blanché H, Passa P, Froguel P, Ruiz J (1997) Paraoxonase polymorphism Met-Leu54 is associated with modified serum concentrations of the enzyme. A possible link between the paraoxonase gene and increased risk of cardiovascular disease in diabetes. J Clin Invest 99:62–66

Garrett IR, Boyce BF, Oreffo RO, Bonewald L, Poser J, Mundy GR (1990) Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. J Clin Invest 85:632–639

Humbert R, Adler DA, Disteche CM, Hassett C, Omiecinski CJ, Furlong CE (1993) The molecular basis of the human serum paraoxonase activity polymorphism. Nature Genet 3:73–76

Johnson ML, Gong G, Kimberling W, Recker SM, Kimmel DB, Recker RB (1997) Linkage of a gene causing high bone mass to human chromosome 11 (11q12–13). Am J Hum Genet 60:1326–1332

Kanis JA, Melton LJ III, Christiansen C, Johnston CC, Khaltaev N (1994) The diagnosis of osteoporosis. J Bone Miner Res 9:1137–1141

Mackness MI, Arrol S, Durrington PN (1991) Paraoxonase prevents accumulation of lipoperoxides in low-density lipoprotein. FEBS Lett 286:152–154

Mackness B, Mackness MI, Arrol S, Turkie W, Durrington PN (1997) Effect of the molecular polymorphisms of human paraoxonase (PON1) on the rate of hydrolysis of paraoxon. Br J Pharmacol 122:265–268

Mackness B, Durrington PN, Abuashia B, Boulton AJ, Mackness MI (2000) Low paraoxonase activity in type II diabetes mellitus complicated by retinopathy. Clin Sci 98:355–363

Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA (1994) Prediction of bone density from vitamin D receptor alleles. Nature 367:284–287

Ota N, Hunt SC, Nakajima T, Suzuki T, Hosoi T, Orimo H, Shirai Y, Emi M (1999) Linkage of interleukin 6 locus to human osteopenia by sibling pair analysis. Hum Genet 105:253–257

Ota N, Nakajima T, Nakazawa I, Suzuki T, Hosoi T, Orimo H, Inoue S, Shirai Y, Emi M (2001) A nucleotide variant in the promoter region of the interleukin-6 gene associated with decreased bone mineral density. J Hum Genet 46:267–272

Parhami F, Morrow AD, Balucan J, Leitinger N, Watson AD, Tintut Y, Berliner JA, Demer LL (1997) Lipid oxidation products have opposite effects on calcifying vascular cell and bone cell differentiation. A possible explanation for the paradox of arterial calcification in osteoporotic patients. Arterioscler Thromb Vasc Biol 17:680–687

Parhami F, Jackson SM, Tintut Y, Le V, Balucan JP, Territo M, demer LL (1999) Atherogenic diet and minimally oxidized low density lipoprotein inhibit osteogenic and promote adipogenic differentiation of marrow stromal cells. J Bone Miner Res 14:2067–2078

Parhami F, Garfinkel A, Demer LL (2000) Role of lipids in osteoporosis. Arterioscler Thromb Vasc Biol 20:2346–2348

Pocock NA, Eisman JA, Hopper JL, Yeates MG, Sambrook PN, Eberl S (1987) Genetic determinations of bone mass in adults: a twin study. J Clin Invest 80:706–710

Primo-Parmo SL, Sorenson RC, Teiber J, La Du BN (1996) The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family. Genomics 33:498–507

Ruiz J, Blanché H, James RW, Garin M-CB, Vaisse C, Charpentier G, Cohen N, Morabia A, Passa P, Froguel P (1995) Gln-Arg192 polymorphism of paraoxonase and coronary artery disease in type 2 diabetes. Lancet 346:869–872

Sanghera DK, Aston CE, Sara N, Kamboh MI (1998) DNA polymorphisms in two paraoxonase genes (PON1 and PON2) are associated with the risk of coronary heart disease. Am J Hum Genet 62:36–44

Serrato M, Marian AJ (1995) A variant of human paraoxonase/arylesterase (HUMPONA) gene is a risk factor for coronary artery disease. J Clin Invest 96:3005–3008

Shimokata H, Ando F, Niino N (2000) A new comprehensive study on aging—the National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS-LSA). J Epidemiol 10: S1–S9

Taboulet J, Frenkian M, Frendo JL, Feingold N, Jullienne A, de Vernejoul MC (1998) Calcitonin receptor polymorphism is associated with a decreased fracture risk in post-menopausal women. Hum Mol Genet 7:2129–2133

Uitterlinden AG, Burger H, Huang Q, Yue F, McGuigan FEA, Grant SFA, Hofman A, van Leeuwen JPTM, Pols HAP, Ralston SH (1998) Relation of alleles of the collagen type Iα1 gene to bone density and the risk of osteoporotic fractures in postmenopausal women. N Engl J Med 338:1016–1021

Yamada Y, Ando F, Niino N, Shimokata H (2001) Transforming growth factor-β1 gene polymorphism and bone mineral density. JAMA 285:167–168

MTHFR Gene Polymorphism as a Risk Factor for Silent Brain Infarcts and White Matter Lesions in the Japanese General Population

The NILS-LSA Study

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Background and Purpose—Silent brain infarcts (SBI) and white matter lesions are relatively common neuroimaging findings, especially in the elderly population. The genetic background for SBI and white matter lesions in a large Japanese general population was investigated.

Methods—Subjects were recruited from participants in the National Institute for Longevity Sciences, Longitudinal Study of Aging. Genotyping of methylenetetrahydrofolate reductase (MTHFR) C677T gene mutation and brain MRI examination were performed in 1721 subjects free of any history of stroke. SBI and white matter lesions were diagnosed from MRI findings.

Results—Of 1721 MRI examinations, SBI was observed in 178 (10.3%). The prevalence of SBI and white matter lesions increased with age. The prevalence of SBI was significantly higher in subjects with the MTHFR TT genotype compared with the TC+CC genotype (14.6% versus 9.5%; 42 of 288 versus 136 of 1433; χ^2=6.71; P=0.010). The stage of white matter lesions was not significantly different. In subjects ≥60 years of age (n=849), the prevalence of SBI was significantly higher in TT than TC+CC (27.7% versus 16.6%; 36 of 130 versus 119 of 719; χ^2=9.16; P=0.002). The prevalence of moderately advanced white matter lesions was also significantly higher in TT than TC+CC (60.7% versus 49.0%; 79 of 130 versus 352 of 719; χ^2=9.16; P=0.002). After correction for other risk factors, the MTHFR TT genotype was independently associated with SBI (odds ratio [OR], 1.72; 95% CI, 1.10 to 2.68; P=0.018) and moderately advanced white matter lesions (OR, 1.58; 95% CI, 1.07 to 2.33; P=0.02).

Conclusions—These findings indicate that the MTHFR TT genotype is an independent risk factor for SBI and white matter lesions in the general Japanese population, especially in elderly subjects. (***Stroke.*** **2003;34:1130-1135.)**

Key Words: amine oxidoreductases ■ brain infarction ■ elderly ■ polymorphism ■ white matter

Silent brain infarcts (SBI) and white matter lesions, which are often incidentally identified during CT or MRI scanning in asymptomatic individuals, are relatively common neuroimaging findings, especially in the elderly population.[1-3] However, the presence of SBI and white matter lesions has been identified as an independent risk factor for the development of future symptomatic stroke[4,5] and dementia.[6] Accordingly, the underlying mechanisms have been the focus of much research.[1-3,7,8] Population-based studies have been performed to identify risk factors for SBI.[2,3] It has been demonstrated that classic risk factors such as hypertension and smoking are involved in the development of SBI.[1-3,7,8] The genetic predisposition to SBI has also been studied.[9,10] However, a population-based study to identify a candidate gene for SBI has not been performed.

Methylenetetrahydrofolate reductase (MTHFR) catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as a methyl donor in the reaction converting homocysteine (Hcy) to methionine.[11,12] An increased plasma Hcy level has consistently been shown to be an independent risk factor for atherosclerotic disorders in several meta-analyses.[11-13] On the other hand, a common mutation of MTHFR, which results in a mild increase in the plasma level of Hcy, has not been reported as a consistent risk factor for atherothrombotic disorders, including stroke.[11,12,14] Although there could be several possible mechanisms underlying the discrepancy, the sampling of cases and controls might account for part of the discrepancy.

Two studies have evaluated the association between MTHFR gene C677T mutations and SBI.[9,10] Both studies

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failed to demonstrate significant associations. One evaluated subjects undergoing medical checkup.[9] To evaluate the genetic predisposition to SBI, community-based sampling of subjects would be less biased in the selection of cases and controls and would eliminate regional differences. Another study evaluated community-dwelling elderly subjects[10]; however, the number of subjects was too small to reach a conclusion. The National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS-LSA) is a comprehensive study on aging in the Japanese general population.[15] In this standardized cohort with >1700 subjects, we investigated the association between MTHFR C677T mutations and the prevalence of SBI. All participants underwent brain MRI examination and were genotyped for the MTHFR C677T mutation.

Methods

Subject Selection

All participants were independent residents in Obu and Higashiura in the Aichi prefecture in central Japan. Residents 40 to 79 years of age were randomly selected from the resident register in cooperation with the local government. They were stratified by both age and sex. Randomly selected men and women were invited by mail to attend an explanatory meeting. At that meeting, the procedures for each examination and the follow-up schedule were fully explained. Written, informed consent to the entire procedure was obtained from each participant. Participants in the present study were recruited from subjects examined in 1997 to 1999. In total, 1758 subjects completed the entire procedure. Among them, 1721 subjects, 876 men and 845 women, free of any history of stroke, including transient ischemic attack, were evaluated in the present study. The ethics committee of the Chubu National Hospital approved all procedures of the NILS-LSA.

Research Area

The residential area of the present study is in the south of Nagoya. It is a commuter town and an industrial area for the Toyota group but still has many orchards and farms; thus, it has both urban and rural characteristics. This research area is in the center of Japan, and the climate is average for Japan. We examined a representative sample of the area's population via a national postal questionnaire of prefecture-stratified random samples of 3000 households from all prefectures in Japan and showed that the lifestyle of this area was the most typical of all areas in Japan.[15]

Risk Factor Evaluation

Details of physical examinations were published elsewhere.[15] In brief, lifestyle, medical history, and prescribed drugs were examined by questionnaires. Anthropometric and blood pressure measurements were performed by a physician. Venous blood was collected early in the morning after at least a 12-hour fast for measurement of serum lipids and plasma glucose. Blood pressure was measured twice at >5-minute intervals in subjects in the sitting position by doctors using a standard sphygmomanometer. The mean of 2 determinations was obtained for each participant. As risk factors for stroke, hypertension, glucose intolerance, hyperlipidemia, and smoking status were evaluated. Hypertension was defined as systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, and/or the use of an antihypertensive drug. Glucose intolerance was defined as fasting plasma glucose ≥110 mg/dL, HbA1c ≥5.8%, and/or the use of medication for lowering blood glucose. Hyperlipidemia was defined as serum total cholesterol ≥230 mg/dL, serum triglycerides ≥170 mg/dL, and/or the use of a lipid-lowering drug.

Brain MRI Examination

Brain MRI imaging was performed in all participants in the present study with a 1.5-T scanner (Toshiba Visart) at the NILS. The head position was oriented in the scanner and stabilized during the scanning procedure by use of a head support. To establish slice orientation, the first scanning sequence consisted of a T1-weighted sagittal series (repetition time [TR], 500 ms; echo time [TE], 15 ms; matrix, 256×256) centered in the midline to define the orbitomeatal line. The second series of T1-weighted axial images (TR, 500 ms; TE, 15 ms; thickness, 8 mm; gap, 1.5 mm; matrix, 256×256) and T2-weighted axial images (TR, 4000 ms; TE, 120 ms; thickness, 8 mm; gap, 1.5 mm; matrix, 320×320) were oriented parallel to the orbitomeatal line. Fourteen slices were taken at each examination.

An infarct was defined as a lesion ≥0.3 cm in diameter shown as a low-signal-intensity area on T1-weighted images that was also visible as a hyperintense lesion on T2-weighted images.[16,17] Small lesions (<1.5 cm) were diagnosed as lacunae. White matter lesions, depicted on T2-weighted images, were classified into 5 grades: grade 0, no abnormality; grade 1, minimal periventricular signal hyperintensities in the form of caps confined exclusively to the anterior horns; grade 2, hyperintensities in both the anterior and posterior horns of the lateral ventricles, periventricular unifocal patches, or rims lining the ventricles; grade 3, multiple periventricular hyperintense punctuate lesions reaching early confluence in the periventricular region; and grade 4, diffuse lesions.[16,17] A neurologist (F.M.) blinded to the clinical status of the subjects interpreted all MRI series.

MTHFR Genotype Analysis

Genomic DNA was extracted from peripheral blood lymphocytes by the standard procedure. MTHFR C677T mutation was determined by allele-specific primer–polymerase chain reaction (ASP-PCR) method. The single nucleotide polymorphism region of the gene was amplified by PCR with 2 ASPs (C-specific primer, 5′-GAAGGTGTCTGCGGGAXCC-3′; T-specific primer, 5′-GAGAAGGTGTCTGCGGGAXTC-3′) and a biotin-labeled common antisense primer (5′-biotin-GAATGTGTCAGCCTCAAAGAAA-3′). Amplified allele-specific DNA products were used for colorimetric genotyping. For MTHFR genotyping, 2 types of wells conjugated with the allele-specific C-type probe (5′-TCTGCGGGAXCCGATTTCAT-3′) or T-type probe (5′-TCTGCGGGAXTCGATTTCAT-3′) were prepared. The amplified DNA product was denatured with NaOH and added to each well. Then, it was hybridized at 37°C for 30 minutes with hybridization buffer containing formamide. After the wells were washed thoroughly, alkaline phosphatase–conjugated streptavidin was added to each well, and the plate was incubated at 37°C for 15 minutes. After the wells were washed, 0.8 mmol/L WST-1[2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-di- sulfophenyl)-2H-tetrazolium, monosodium salt] and 0.4 mmol/L BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt), a substrate for alkaline phosphatase, was added, and colorimetry was performed. The genotypes were identified by the absorbance signal ratio between C type–specific and T type–specific wells. The validity of the ASP-PCR method was confirmed with genotyped DNA samples obtained by the standard method reported by Frosst et al.[18] KOD polymerase derived from Thermococcus kodakaraensis KOD1 was used.[19] The fidelity of our method is 3.4 times higher than that with *Taq* DNA polymerase. The mutation rate with this method is 0.35%.[19]

Statistical Analysis

All values are expressed as mean±SD if not specified. The associations between SBI and white matter lesions and MTHFR genotype were analyzed by χ^2 test. Logistic regression analysis was used to explore the independence of the effect of the MTHFR TT genotype on the prevalence of SBI and the presence of white matter lesions. All statistical analyses were performed with SAS software (SAS Institute Inc). A value of $P<0.05$ was considered statistically significant.

TABLE 1. Background Characteristics of Subjects With Silent Brain Infarcts and Moderate White Matter Lesions

	Silent Brain Infarcts		White Matter Lesions	
	(−)	(+)	Grades 0–1	Grades 2–4
n	1543	178	1225	496
Male, %	50	64†	51	52
Age, y	58±11	69±8‡	55±10	68±8‡
BMI, kg/m²	22.9±3.0	23.2±3.3	23.0±3.0	22.8±4.1
SBP, mm Hg	123±19	134±19‡	122±19	130±21‡
DBP, mm Hg	75±11	80±11‡	75±11	78±12‡
T-Chol, mg/dL	220±35	220±34	219±35	222±36
HDL-Chol, mg/dL	62±15	59±17*	62±16	61±15
Triglyceride, mg/dL	122±87	126±60	123±93	122±61
Blood glucose, mg/dL	103±21	108±23*	103±21	105±22
Hypertension, %	31	66‡	27	51‡
Hyperlipidemia, %	48	52	46	54*
Glucose intolerance, %	21	35‡	21	28*
Current smoker, %	23	24	24	21

MTHFR indicates methylenetetrahydrofolate reductase; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; T-Chol, total cholesterol; HDL-Chol, high density lipoprotein cholesterol.

Values are mean±SD. *P<0.05, †P<0.001, ‡P<0.0001 versus corresponding controls [silent brain infarcts (−) and white matter lesions 0–1].

Results

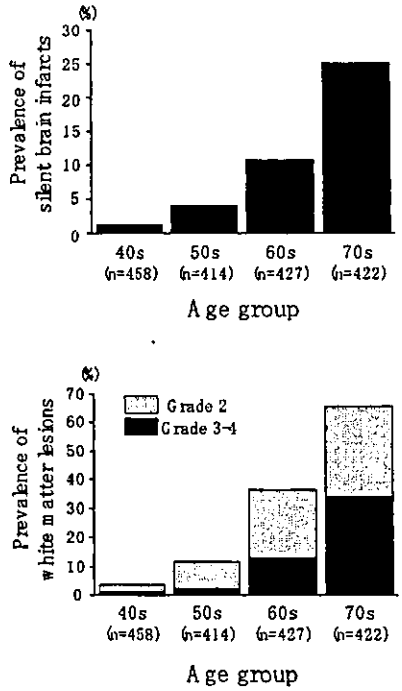
Background Characteristics of Participants and Prevalence of SBI

Table 1 summarizes the backgrounds of all participants subdivided by the presence of SBI and moderately advanced white matter lesions. The presence of SBI and white matter lesions was associated with advanced age, hypertension, and glucose intolerance. The prevalence of SBI and white matter lesions significantly increased with age (the Figure). In subjects ≥60 years of age, SBI was observed in 18.3% (155 of 849).

MTHFR Gene Mutation and SBI and White Matter Lesions

The breakdown of the total 1721 subjects by MTHFR C677T gene mutation was as follows: CC genotype, 623; CT genotype, 810; and TT genotype, 288. The distribution of MTHFR genotypes was consistent with published reports on Japanese subjects[18–20] and was in agreement with the Hardy-Weinberg proportion (P=0.80).

The prevalence of SBI and the grade of white matter lesions are shown in Table 2. The prevalence of SBI was significantly higher in subjects with the MTHFR TT genotype compared with C carriers (CT+CC). However, there was no significant association between MTHFR genotype and grade of white matter lesions. Because SBI and white matter lesions were prevalent only after the age of 60 years, the associations between MTHFR genotype and prevalence of SBI and white matter lesions were evaluated in subjects ≥60 years of age. In this population, the MTHFR genotype



Age-dependent increases in prevalence of SBI and white matter lesions. Prevalence of both types of lesion increased with age.

was significantly associated with the presence of SBI and white matter lesions (Table 3).

To further investigate whether the MTHFR genotype is associated with SBI independently of other known risk factors, logistic regression analysis for SBI was performed with 3 models including the following risk factors; hypertension, hyperlipidemia, glucose intolerance, smoking, and

TABLE 2. Prevalence of Silent Brain Infarcts and White Matter Lesions in 3 MTHFR Genotypes in Total Subjects

MTHFR (n)	CC (623)	CT (810)	TT (288)	CC+CT (1433)	Total (1721)
No SBI	568	729	246	1297	1543
SBI	55	81	42	136	178
		χ^2=7.23		χ^2=6.71*	
		P=0.027		P=0.010	
Lacunae	51	68	37	119	156
		χ^2=6.21		χ^2=6.17*	
		P=0.045		P=0.013	
White matter lesions					
Grades 0–1	455	571	199	1026	1225
Grade 2	93	143	51	236	287
Grades 3–4	75	96	38	171	209
		χ^2=2.65		χ^2=0.75*	
		P=0.62		P=0.69	
Grades 2–4	168	239	89	407	496
		χ^2=1.84		χ^2=0.73*	
		P=0.40		P=0.39	

SBI indicates silent brain infarct.

*Comparison with MTHFR TT genotype.

TABLE 3. Prevalence of Silent Brain Infarcts and White Matter Lesions in 3 MTHFR Genotypes in Subjects Aged 60 or Over

MTHFR (n)	CC (304)	CT (415)	TT (130)	CC+CT (719)	Total (849)
No SBI	257	343	94	600	694
SBI	47	72	36	119	155
		χ^2=9.58		χ^2=9.16*	
		P=0.008		P=0.002	
Lacunae	44	63	32	107	139
		χ^2=8.08		χ^2=8.10*	
		P=0.018		P=0.004	
White matter lesions					
Grades 0–1	155	212	51	367	418
Grade 2	78	114	42	192	234
Grades 3–4	71	89	37	160	197
		χ^2=6.69		χ^2=6.20*	
		P=0.153		P=0.045	
Grades 2–4	149	203	79	352	431
		χ^2=6.15		χ^2=6.15*	
		P=0.046		P=0.013	

SBI indicates silent brain infarct.
*Comparison with MTHFR TT genotype.

MTHFR genotype in subjects ≥60 years of age (Table 4). It revealed that the MTHFR TT genotype was independently associated with SBI, asymptomatic lacunar infarctions, and white matter lesions of grade 2 or higher.

Discussion

Meta-analyses have revealed a consistent association between the plasma level of Hcy and atherosclerotic disorders.[11,13] Boushey et al[13] reported that the odds ratio (OR) for coronary arterial disease with a 5-μmol/L Hcy increment was 1.6 (95% CI, 1.4 to 1.7) for men and 1.8 (95% CI, 1.3 to 1.9) for women in their meta-analysis. Recently, it has also been reported that a high Hcy level was significantly associated with SBI and white matter lesions.[10,20] Although MTHFR C677T mutation is a major cause of mild hyperhomocysteinemia, Brattstrom et al[14] showed in their meta-analysis that the mutation did not increase cardiovascular risk in their 5869 controls and 6644 cases. They suggested that the modest increase in plasma concentration of Hcy found in patients with cardiovascular disease is an epiphenomenon, a consequence of the effects of the well-established standard risk factors for vascular disease and renal function, and that it is not directly causal. However, Ueland et al[11] further extended the interpretation of the findings of meta-analysis. Because the estimated relative risk for atherosclerotic disorders associated with a modest increase in plasma Hcy (2.6 μg/mL) by MTHFR TT mutation is 1.10 to 1.15, it is expected that a larger population would be necessary to prove a significant association with the MTHFR TT genotype.[11] These findings indicate that the genetic association between MTHFR TT genotype and atherosclerotic disorders is not conclusive.

The association between the MTHFR gene mutation and SBI has been studied in the Japanese population.[9,10] Those studies reported a lack of association between MTHFR and SBI. In a study by Notsu et al,[9] SBI patients were recruited from consecutive patients who underwent brain MRI examination for a health screening examination. Accordingly, these subjects were neither randomly selected nor community-based samples. One of the concerns in the recruitment of participants in medical checkups is that the possibility that some could have subtle symptoms prompting them to have a medical checkup, including brain MRI, cannot be excluded. To evaluate the genetic predisposition to SBI, community-based sampling of subjects would be less biased in the selection of cases and controls and would eliminate regional differences. Another study reported by Matsui et al[10] was a community-based study. Although they did not find a positive association, the number (38 cases) was too small to reach a conclusion. To respond to these concerns, large, population-based, random sampling is advisable. NILS-LSA is a community-based random sample study in central Japan. Our preliminary study indicated that the area is representative of the total Japanese population. Region-dependent differences in folic acid[21] could also be eliminated. All participants underwent brain MRI, and genotyping was performed. The advantage of a community-dwelling study is minimal bias in the selection of cases and controls. On the other hand, a disadvantage is that the number of cases of concern is small,

TABLE 4. Odds Ratio for Presence of Silent Brain Infarcts, Lacunar Lesions, and White Matter Lesions in Subjects Aged 60 or Over

	Model I		Model II		Model III	
	OR	CI	OR	CI	OR	CI
SBI	1.93	1.25–2.97	1.82	1.18–2.82	1.72	1.10–2.68
	P=0.0028		P=0.0073		P=0.018	
Lacunae	1.91	1.22–3.00	1.81	1.15–2.86	1.71	1.08–2.73
	P=0.0049		P=0.011		P=0.024	
White matter lesions						
Grades 2–4	1.62	1.10–2.37	1.63	1.11–2.39	1.58	1.07–2.33
	P=0.013		P=0.013		P=0.02	

OR indicates odds ratio; CI, 95% confidence interval; SBI, silent brain infarct. Model I, no correction for other risk factors; model II, correction for sex, smoking, hyperlipidemia, and glucose intolerance; model III, correction for sex, smoking, hyperlipidemia, glucose intolerance, and hypertension.

and a large population is necessary to have enough cases for analysis. One way to overcome this disadvantage is to use a surrogate condition such as SBI for the cases.

Asymptomatic brain infarction is reported to be common in the elderly population. Although neurologically asymptomatic, MRI-proven abnormal findings have been shown to be associated with several disorders.[16,17,22–25] Accordingly, it is conceivable that the subjects with SBI might not have been completely free of symptoms in the present study. In our previous studies, SBI was shown to be associated with hypertensive end-organ damage[24] and abnormal diurnal changes in blood pressure.[17,25] Although we did not analyze cognitive function in the present study, an association between impaired cognitive function and SBI and white matter lesions has also been reported.[14,22] In several studies, the MTHFR TT genotype has also been demonstrated to be a risk factor for dementia,[26,27] although conflicting results have also been reported.[28,29] These findings suggest a possible association between the MTHFR TT genotype and cognitive impairment in the general population. However, this issue needs to be confirmed in a large community population.

Although the origin of the TT variant is not known, the high prevalence of this polymorphism in most populations could indicate that the TT variant might represent an ancestral genetic adaptation to living.[12] Enhanced homeostasis, cell proliferation, and tissue repair have been postulated as underlying mechanisms.[12] The MTHFR TT variant shows reduced enzymatic activity, resulting in an increase in Hcy concentration. It has been reported that hyperhomocysteinemia is a possible causal factor in free radical generation during the acute phase of thrombotic cerebrovascular stroke.[30] Recently, it has also been reported that the MTHFR TT genotype was associated with reduced superoxide dismutase activity.[31] Because DNA can be damaged in oxidative stress such as ischemia-reperfusion injury in the brain, DNA repair after oxidative stress could also be altered by the TT mutation. Furthermore, it has been reported that errors in DNA repair caused by oxidative stress occur preferentially at GC sequences in the brain,[32,33] suggesting that the TT mutation could be a hallmark of oxidative stress in the brain. These findings suggest that damage caused by oxidative stress such as reperfusion could be enhanced in subjects with the MTHFR TT mutation, which may account for their higher prevalence of SBI. However, this hypothesis requires further study.

In summary, in a large, randomly selected, community-based population, the MTHFR TT genotype was an independent risk factor for both SBI and white matter lesions. Because the prevalence of SBI is relatively high, an interventional approach could be useful in reducing the future risk of developing symptomatic stroke and cognitive decline.

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References

1. Shinkawa A, Ueda K, Kiyohara Y, Kato I, Sueishi K, Tsuneyoshi M, Fujishima M. Silent cerebral infarction in a community-based autopsy series in Japan: the Hisayama Study. *Stroke*. 1995;26:380–385.
2. Longstreth WT Jr, Bernick C, Manolio TA, Bryan N, Jungreis CA, Price TR. Lacunar infarcts defined by magnetic resonance imaging of 3660 elderly people: the Cardiovascular Health Study. *Arch Neurol*. 1998;55: 1217–1225.
3. Vermeer SE, Koudstaal PJ, Oudkerk M, Hofman A, Breteler MM. Prevalence and risk factors of silent brain infarcts in the population-based Rotterdam Scan Study. *Stroke*. 2002;33:21–25.
4. Kobayashi S, Okada K, Koide H, et al. Subcortical silent brain infarction as a risk factor for clinical stroke. *Stroke*. 1997;28:1932–1939.
5. Bernick C, Kuller L, Dulberg C, Longstreth WT Jr, Manolio T, Beauchamp N, Price T, for the Cardiovascular Health Study Collaborative Research Group. Silent MRI infarcts and the risk of future stroke: the Cardiovascular Health Study. *Neurology*. 2001;57:1222–1229.
6. Kuller LH, Shemanski L, Manolio T, et al. Relationship between ApoE, MRI findings, and cognitive function in the Cardiovascular Health Study. *Stroke*. 1998;29:388–398.
7. Howard G, Wagenknecht LE, Cai J, Cooper L, Kraut MA, Toole JF. Cigarette smoking and other risk factors for silent cerebral infarction in the general population. *Stroke*. 1998;29:913–917.
8. Vermeer SE, van Dijk EJ, Koudstaal PJ, Oudkerk M, Hofman A, Clarke R, Breteler MM. Homocysteine, silent brain infarcts, and white matter lesions: the Rotterdam Scan Study. *Ann Neurol*. 2002;51:285–289.
9. Notsu Y, Nabika T, Park H-Y, Masuda J, Kobayashi S. Evaluation of genetic risk factors for silent brain infarction. *Stroke*. 1999;30: 1881–1886.
10. Matsui T, Arai H, Yuzuriha T, Yao H, Miura M, Hashimoto S, Higuchi S, Matsushita S, Morikawa M, Kato A, Sasaki H. Elevated plasma homocysteine levels and risk of silent brain infarction in elderly people. *Stroke*. 2001;32:1116–1119.
11. Ueland M, Refsum H, Beresford SAA, Vollset SE. The controversy over homocysteine and cardiovascular risk. *Am J Clin Nutr*. 2000;72:324–332.
12. Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE. Biological and clinical implications of the MTHFR C677T polymorphism. *Trends Pharmacol Sci*. 2001;22:195–201.
13. Boushey CJ, Beresford SA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: probable benefits of increasing folic acid intakes. *JAMA*. 1995;274: 1049–1057.
14. Brattstrom L, Wilcken DE, Ohrvik J, Brudin L. Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease: the result of a meta-analysis. *Circulation*. 1998;98:2520–2526.
15. Shimokata H, Ando F, Niino N. A new comprehensive study on aging: the National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS-LSA). *J Epidemiol*. 2000;10(suppl):S1–S9.
16. Matsubayashi K, Okumiya K, Wada T, Osaki Y, Fujisawa M, Doi Y, Ozawa T. Postural dysregulation in systolic blood pressure is associated with worsened scoring on neurobehavioral function tests and leukoariosis in the older elderly living in a community. *Stroke*. 1997;28:2169–2173.
17. Kohara K, Jiang YN, Igase M, Takata Y, Fukuoka T, Okura T, Kitami Y, Hiwada K. Postprandial hypotension is associated with asymptomatic cerebrovascular damage in essential hypertensive patients. *Hypertension*. 1999;33:565–568.
18. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJH, den Heijer M, Kluijtmans LAJ, van den Hauvel LP, Rozen R. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet*. 1995;10:111–113.
19. Takagi M, Nishioka M, Kakihara H, Kitabayashi M, Inoue H, Kawakami B, Oka M, Imanaka T. Characterization of DNA polymerase from Pyrococcus sp. strain KOD1 and its application to PCR. *Appl Environ Microbiol*. 1997;63:4504–4510.
20. Vermeer SE, van Dijk EJ, Koudstaal PJ, Oudkerk M, Hofman A, Clarke R, Breteler MM. Homocysteine, silent brain infarcts, and white matter lesions: the Rotterdam Scan Study. *Ann Neurol*. 2002;51:285–289.
21. Dekou V, Whincup P, Papacosta O, Ebrahim S, Lennon L, Ueland PM, Refsum H, Humphries SE, Gudnason V. The effect of the C677T and A1298C polymorphisms in the methylenetetrahydrofolate reductase gene on homocysteine levels in elderly men and women from the British Regional Heart Study. *Atherosclerosis*. 2001;154:659–666.
22. Price TR, Manolio TA, Kronmal RA, Kittner SJ, Yue NC, Robbins J, Anton-Culver H, O'Leary DH. Silent brain infarction on magnetic resonance imaging and neurological abnormalities in community-dwelling older adults: the Cardiovascular Health Study (CHS) Collaborative Research Group. *Stroke*. 1997;28:1158–1164.

23. Sato R, Bryan RN, Fried LP. Neuroanatomic and functional correlates of depressed mood: the Cardiovascular Health Study. *Am J Epidemiol.* 1999;150:919–929.
24. Kohara K, Zhao B, Jiang YN, Takata Y, Fukuoka T, Igase M, Miki T, Hiwada K. Relation of left ventricular hypertrophy and geometry to asymptomatic cerebrovascular damage in essential hypertension. *Am J Cardiol.* 1999;83:367–370.
25. Kohara K, Igase M, Jiang YN, Fukuoka T, Maguchi M, Okura T, Kitami Y, Hiwada K. Asymptomatic cerebrovascular damages in essential hypertension in the elderly. *Am J Hypertens.* 1997;10:829–835.
26. Yoo J-H, Choi G-D, Kang S-S. Pathogenicity of thermolabile methylenetetrahydrofolate reductase for vascular dementia. *Arterioscler Thromb Vasc Biol.* 2000;20:1921–1925.
27. Nishiyama M, Kato Y, Hashimoto M, Yukawa S, Omori K. Apolipoprotein E, methylenetetrahydrofolate reductase (MTHFR) mutation and the risk of senile dementia: an epidemiological study using the polymerase chain reaction (PCR) method. *J Epidemiol.* 2000;10:163–172.
28. Gussekloo J, Heijmansb BT, Slagboomb PE, Lagaaya AM, D L Knooka DL, Westendorp RGJ. Thermolabile methylene tetrahydrofolate reductase gene and the risk of cognitive impairment in those over 85. *J Neurol Neurosurg Psychiatry.* 1999;67:535–538.
29. Pollak RD, Pollak A, Idelson M, Bejarano-Achache I, Doron D, Blumenfeld A. The C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene and vascular dementia. *J Am Geriatr Soc.* 2000;48:664–668.
30. El Kossi MM, Zakhary MM. Oxidative stress in the context of acute cerebrovascular stroke. *Stroke.* 2000;31:1889–1892.
31. Mayer O Jr, Äimon J, Rosolová H, Hromádka M, Äubrt I, Vobrubová I. The effects of folate supplementation on some coagulation parameters and oxidative status surrogates. *Eur J Clin Pharmacol.* 2002;58:1–5.
32. Liu PK, Hsu CY, Dizdaroglu M, Floyd RA, Kow YW, Karakaya A, Rabow LE, Cui JK. Damage, repair, and mutagenesis in nuclear genes after mouse forebrain ischemia-reperfusion. *J Neurosci.* 1996;16:6795–6806.
33. Liu PK, Grossman RG, Hsu CY, Robertson CS. Ischemic injury and faulty gene transcripts in the brain. *Trends Neurosci.* 2001;24:581–588.

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Association of polymorphisms of the osteoprotegerin gene with bone mineral density in Japanese women but not men

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Abstract

Given that osteoprotegerin plays an important role in bone remodeling, the osteoprotegerin gene may be a candidate locus for susceptibility to osteoporosis. The relation of polymorphisms in the promoter of the osteoprotegerin gene to bone mineral density (BMD) was examined in a Japanese population-based prospective cohort study with randomly recruited subjects (1095 women and 1125 men for the 950T → C polymorphism, 1094 women and 1127 men for the 245T → G polymorphism). BMD at the radius was measured by peripheral quantitative computed tomography, and that for the total body, lumbar spine, right femoral neck, right trochanter, and right Ward's triangle was measured by dual-energy X-ray absorptiometry. Genotypes were determined with a fluorescence-based allele-specific DNA primer assay system. Among 950T → C genotypes, BMD for the proximal radius was lower in premenopausal women with the *CC* genotype than in those with the *TT* or *TC* genotype; the difference in BMD between the two groups was 3.9% ($P = 0.0075$). Among 245T → G genotypes, BMD for the radius, total body, femoral neck, trochanter, and Ward's triangle was lower in postmenopausal women with the *GG* genotype than in those with the *TT* or *TG* genotype, the *TT* genotype, or the *TG* genotype; the differences in BMD between the *GG* genotype and the *TT* or *TG* genotype were 19.8% for the distal radius ($P = 0.0015$), 13.1% for the proximal radius ($P = 0.0095$), 11.2% for the total body ($P = 0.0013$), 12.9% for the femoral neck ($P = 0.0067$), 18.7% for the trochanter ($P = 0.0008$), and 27.1% for Ward's triangle ($P = 0.0038$). BMD was not associated with the 950T → C or 245T → G genotypes in men. The present results implicate the osteoprotegerin gene as a susceptibility locus for reduced BMD in Japanese women.

Keywords: Bone mineral density; Genetics; Osteoporosis; Osteoprotegerin; Osteoclastogenesis inhibitory factor; Polymorphism

Introduction

Osteoporosis, a major health problem of the elderly, is characterized by a reduction in bone mineral density (BMD) and a deterioration in the microarchitecture of bone, both of which result in predisposition to fractures [1]. Although several environmental factors, including diet, smoking, and physical exercise, influence BMD, a genetic contribution to this parameter has been recognized [2]. Genetic linkage analyses [3–5] and candidate gene association studies [5–7] have thus implicated several loci and candidate genes in the regulation of bone mass and the prevalence of osteoporosis or osteoporotic fractures. The genes that contribute to genetic susceptibility to osteoporosis, however, remain to be fully characterized.

Osteoprotegerin is a soluble member of the tumor necrosis factor (TNF) receptor superfamily of proteins. In vitro studies suggest that osteoprotegerin inhibits osteoclastogenesis by interrupting intercellular signaling between osteoblastic stromal cells and osteoclast progenitors [8]. Osteoprotegerin-deficient mice exhibit a condition similar to juvenile Paget's disease characterized by a marked decrease in trabecular and cortical bone density, pronounced thinning of the parietal bone of the skull, and a high incidence of fractures [9], whereas hepatic expression of osteoprotegerin in transgenic mice results in osteopetrosis and a coincident decrease in the proportion of osteoclasts at the later stages

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of differentiation [8]. The systemic administration of recombinant osteoprotegerin also results in a marked increase in BMD in normal rats as well as in prevention of bone loss in ovariectomized rats [8,10]. Furthermore, a single subcutaneous injection of osteoprotegerin reduced bone resorption in postmenopausal women [11]. Similar treatment with a recombinant osteoprotegerin construct (AMGN-0007) suppressed bone resorption in patients with multiple myeloma or breast cancer with bone metastases [12]. Given the importance of osteoprotegerin in bone remodeling, the osteoprotegerin gene (*OPG*) may be a candidate locus for susceptibility to osteoporosis.

Several single nucleotide polymorphisms (SNPs) have been detected in *OPG*, some of which have been shown to be associated with BMD in postmenopausal women [13–15] or with osteoporotic fractures in women and men [16]. Given the ethnic divergence of gene polymorphisms, however, it is important to examine polymorphisms potentially related to BMD in each ethnic group. We have now examined whether the 950T → C and 245T → G SNPs in the promoter of *OPG* are associated with BMD in Japanese women or men in a population-based study.

Materials and methods

Study population

The present study represents a cross-sectional analysis in a population-based prospective cohort study of aging and age-related diseases [17,18]. The subjects are stratified by both age and gender, and are randomly selected from resident registrations in the city of Obu and town of Higashiura in central Japan. The lifestyle of residents in this area is typical of that of individuals in most regions of Japan. The numbers of men and women recruited are similar and the baseline age is 40–79 years, with similar numbers of participants in each decade (40s, 50s, 60s, and 70s). The subjects will be followed up every 2 years. All participants are subjected at a special center to a detailed examination, which includes not only medical evaluation, but also assessment of exercise physiology, body composition, nutrition, and psychology. Individuals with disorders known to cause abnormalities of bone metabolism, including diabetes mellitus, renal diseases, rheumatoid arthritis, as well as thyroid, parathyroid, and other endocrinologic diseases, were excluded from the present study. Women who had taken drugs, such as estrogen, progesterone, glucocorticoids, and bisphosphonates were also excluded. We examined the relation of BMD at various sites to the 950T → C SNP of *OPG* in 2220 participants (1095 women, 1125 men) and to the 245T → G SNP in 2221 participants (1094 women, 1127 men). The study protocol was approved by the relevant committee on the ethics of human research and written informed consent was obtained from each subject.

Measurement of BMD

BMD at the radius was measured by peripheral quantitative computed tomography (pQCT) (Desiscan 1000; Scanco Medical, Bassersdorf, Switzerland) and was expressed as D50 (distal radius BMD for the inner 50% of the cross-sectional area, comprising mostly cancellous bone), D100 (distal radius BMD for the entire cross-sectional area, including both cancellous and cortical bone), and P100 (proximal radius BMD for the entire cross-sectional area, consisting mostly of cortical bone). BMD for the total body, lumbar spine (L2–L4), right femoral neck, right trochanter, and right Ward's triangle was measured by dual-energy X-ray absorptiometry (DXA) (QDR 4500; Hologic, Bedford, MA). The coefficients of variance of the pQCT instrument for BMD values were 0.7% (D50), 1.0% (D100), and 0.6% (P100), and those of the DXA instrument were 0.9% (total body), 0.9% (L2–L4), 1.3% (femoral neck), 1.0% (trochanter), and 2.5% (Ward's triangle).

Determination of genotypes

Genotypes were determined with a fluorescence-based allele-specific DNA primer assay system (Toyobo Gene Analysis, Tsuruga, Japan) [19]. The polymorphic regions of *OPG* were amplified by the polymerase chain reaction with allele-specific sense primers labeled at the 5′ end with either fluorescein isothiocyanate (5′-CAGC CCTGAAAGCGTTAXTC-3′ for 950T → C and 5′-CG CTGAACTTCTGGAGXAG-3′ for 245T → G) or Texas red (5′-CAGCCCTGAAAGCGTTAXCC-3′ for 950T → C and 5′-CGCTGAACTTCTGGAGXCG-3′ for 245T → G) and with an antisense primer labeled at the 5′ end with biotin (5′-GGGTGTGCAGAAAGCTC CA-3′ for 950T → C and 5′-GCTTGAGGCTAGTGGA AAGAC-3′ for 245T → G). The reaction mixtures (25 μL) contained 20 ng DNA, 5 pmol each primer, 0.2 mmol/L each deoxynucleoside triphosphate, 2.5 mmol/L $MgCl_2$, and 1 U rTaq DNA polymerase (Toyobo, Osaka, Japan) in polymerase buffer. The amplification protocol comprised initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 62.5 °C (950T → C) or 60 °C (245T → G) for 30 s, and extension at 68 °C for 30 s; and a final extension at 68 °C for 2 min.

The amplified DNA was incubated in a solution containing streptavidin-conjugated magnetic beads in the wells of a 96-well plate at room temperature. The plate was then placed on a magnetic stand and the supernatants from each well were transferred to the wells of a 96-well plate containing 0.01 mol/L NaOH and were measured for fluorescence with a microplate reader

(Fluoroscan Ascent; Dainippon Pharmaceutical, Osaka, Japan) at excitation and emission wavelengths of 485 and 538 nm, respectively, for fluorescein isothiocyanate and of 584 and 612 nm, respectively, for Texas red.

Statistical analysis

Quantitative data were compared among three groups by one-way analysis of variance and the Tukey–Kramer post hoc test, and between two groups by the unpaired Student's t test. Allele frequencies were estimated by the gene-counting method, and the χ^2 test was used to identify significant departure from Hardy–Weinberg equilibrium. Unless indicated otherwise, a P value of <0.05 was considered statistically significant.

Results

The distribution of combined genotypes for the 950T→C and 245T→G SNPs of *OPG* is shown in Table 1. The distribution of haplotypes was as follows: *950T/245T*, 56.1%; *950T/245G*, 4.1%; *950C/245T*, 32.9%; and *950C/245G*, 6.9%. The two SNPs exhibited marked linkage disequilibrium [pairwise linkage disequilibrium coefficient, D' (D/D_{max}), of 0.3858 and standardized linkage disequilibrium coefficient, r, of 0.1664; $P < 0.0001$].

To examine the possible influence of menopause on the relation between genotype and BMD, we analyzed BMD and other characteristics for premenopausal and postmenopausal women independently. The distributions of 950T→C genotypes of *OPG* were in Hardy–Weinberg equilibrium for both premenopausal and postmenopausal women (Table 2). Age for premenopausal or postmenopausal women and years since menopause for postmenopausal women did not differ among 950T→C genotypes. Given the multiple comparisons of genotypes with BMD values, we considered a P value of <0.01 to be significant for such associations. On the basis of this criterion, for premenopausal women, BMD at the proximal radius (P100) was significantly lower in those with the *CC* genotype than in those with the *TT* or *TC* genotype. The difference in P100 between the *CC* genotype and the *TT* or *TC* genotype (expressed as a percentage of the corresponding larger value) in premenopausal women was 3.9% ($P = 0.0075$). In contrast, for postmenopausal women, BMD was not associated with 950T→C genotype (Table 2). For men, the distribution of 950T→C ge-

Table 1
Distribution of the 950TC and 245T→G genotypes of *OPG* among study subjects

245T→G genotype	950T→C genotype			Total
	TT	*TC*	*CC*	
TT	804 (36.05%)	785 (35.20%)	193 (8.65%)	1782 (79.91%)
TG	0 (0%)	284 (12.74%)	135 (6.05%)	419 (18.79%)
GG	0 (0%)	0 (0%)	29 (1.30%)	29 (1.30%)
Total	804 (36.05%)	1069 (47.94%)	357 (16.01%)	2230 (100%)

Table 2
BMD and other characteristics of women ($n = 1095$) according to menopausal status and the 950T→C genotype of *OPG*

Characteristic	Premenopausal women ($n = 279$)				Postmenopausal women ($n = 816$)			
	TT	*TC*	*TT + TC*	*CC*	*TT*	*TC*	*TT + TC*	*CC*
Number (%)	101 (36.2)	131 (47.0)	232 (83.2)	47 (16.8)	295 (36.2)	387 (47.4)	682 (83.6)	134 (16.4)
Age (years)	46.1 ± 0.5	46.3 ± 0.4	46.2 ± 0.3	46.1 ± 0.7	64.3 ± 0.5	63.8 ± 0.4	64.0 ± 0.3	63.7 ± 0.7
Years since menopause					15.4 ± 0.5	14.7 ± 0.5	15.0 ± 0.4	14.7 ± 0.8
BMD values measured by pQCT (mg/cm³)								
D50	248.2 ± 5.8	245.7 ± 5.2	246.8 ± 3.8	237.5 ± 8.4	163.3 ± 4.1	162.4 ± 3.6	162.8 ± 2.7	166.8 ± 6.1
D100	606.3 ± 8.1	609.0 ± 7.3	607.8 ± 5.4	591.8 ± 11.8	436.9 ± 6.4	444.1 ± 5.6	441.0 ± 4.2	451.7 ± 9.6
P100	1368.4 ± 12.5*	1369.5 ± 11.2*	1369.0 ± 8.3†	1315.2 ± 18.2	1064.0 ± 11.1	1085.0 ± 9.8	1075.9 ± 7.3	1093.7 ± 16.6
BMD values measured by DXA (g/cm²)								
Total body	1.084 ± 0.009	1.093 ± 0.008	1.090 ± 0.006	1.113 ± 0.013	0.917 ± 0.006	0.920 ± 0.006	0.919 ± 0.004	0.919 ± 0.010
L2–L4	1.018 ± 0.012	1.026 ± 0.011	1.023 ± 0.008	1.033 ± 0.018	0.802 ± 0.009	0.813 ± 0.008	0.808 ± 0.006	0.815 ± 0.013
Femoral neck	0.771 ± 0.010	0.767 ± 0.009	0.769 ± 0.007	0.788 ± 0.014	0.641 ± 0.006	0.644 ± 0.005	0.643 ± 0.004	0.650 ± 0.009
Trochanter	0.656 ± 0.009	0.654 ± 0.008	0.655 ± 0.006	0.674 ± 0.013	0.540 ± 0.006	0.537 ± 0.005	0.538 ± 0.004	0.547 ± 0.009
Ward's triangle	0.650 ± 0.013	0.659 ± 0.011	0.655 ± 0.009	0.680 ± 0.019	0.442 ± 0.009	0.457 ± 0.008	0.451 ± 0.006	0.462 ± 0.013

Data are means ± SE. pQCT, peripheral quantitative computed tomography; DXA, dual-energy X-ray absorptiometry.
* $P < 0.05$.
† $P < 0.01$ versus *CC*.

notypes was in Hardy–Weinberg equilibrium, but BMD did not differ among these genotypes (data not shown).

The distributions of genotypes for the 245T → G SNP of *OPG* were in Hardy–Weinberg equilibrium in both premenopausal and postmenopausal women (Table 3). Age did not differ among 245T → G genotypes for premenopausal women. For postmenopausal women, age and years since menopause were greater in individuals with the *GG* genotype than in those with the *TT* or the *TG* genotypes. For premenopausal women, BMD was not associated with 245T → G genotype. On the basis of the criterion of $P < 0.01$, for postmenopausal women, BMD for the distal (D100) or proximal (P100) radius, total body, femoral neck, trochanter, or Ward's triangle was significantly lower in those with the *GG* genotype than in those with the *TT* or *TG* genotype, with the *TT* genotype, or with the *TG* genotype (Table 3). The differences in BMD between the *GG* genotype and the *TT* or *TG* genotype in postmenopausal women were 19.8% for D100 ($P = 0.0015$), 13.1% for P100 ($P = 0.0095$), 11.2% for the total body ($P = 0.0013$), 12.9% for the femoral neck ($P = 0.0067$), 18.7% for the trochanter ($P = 0.0008$), and 27.1% for Ward's triangle ($P = 0.0038$). For men, the distribution of genotypes for the 245T → G SNP was in Hardy–Weinberg equilibrium, but BMD did not differ among these genotypes (data not shown).

Discussion

Given that selection bias can influence the results of association studies, it is important that study populations be genetically and ethnically homogeneous. Our study population was recruited randomly from individuals resident in Obu city and Higashiura town in central Japan, where the population is thought to share the same ethnic ancestry and to possess a homogeneous genetic background [17]. We also showed that the genotype distributions for both of the SNPs studied were in Hardy–Weinberg equilibrium both in women and in men. Our study population therefore appeared genetically homogeneous, and we thus appeared to avoid admixture and selection bias.

Osteoclastogenesis is regulated by three TNF- or TNF receptor-related proteins: receptor activator of nuclear factor-κB (RANK) [20,21], RANK ligand (RANKL) [22,23], and osteoprotegerin [8,10]. RANKL expressed on the surface of bone marrow stromal cells induces the differentiation of osteoclasts, enhances the activity of mature osteoclasts, and inhibits osteoclast apoptosis by binding to its functional receptor, RANK, expressed on osteoclasts or their progenitors [22–26]. The interaction between RANKL and RANK is antagonized by osteoprotegerin, which acts as a decoy receptor for RANKL. The biological effects of osteoprotegerin include inhibition of the later stages of osteoclastogenesis [8,10,27], suppression of the activation of mature osteoclasts [22,25,28], and induction of osteoclast apoptosis [29]. The balance between osteoprotegerin and RANKL may thus represent an important determinant of bone resorption [27]. The importance of osteoprotegerin in the regulation of bone remodeling in humans has been indicated by the occurrence of juvenile Paget's disease, characterized by rapid remodeling of

Table 3
BMD and other characteristics of women ($n = 1094$) according to menopausal status and the 245T → G genotype of *OPG*

Characteristic	Premenopausal women ($n = 276$)				Postmenopausal women ($n = 818$)			
	TT	*TG*	*TT + TG*	*GG*	*TT*	*TG*	*TT + TG*	*GG*
Number (%)	225 (81.5)	49 (17.8)	274 (99.3)	2 (0.7)	637 (77.9)	169 (20.7)	806 (98.5)	12 (1.5)
Age (years)	46.2 ± 0.3	46.1 ± 0.7	46.2 ± 0.3	49.0 ± 3.2	63.9 ± 0.3*	63.7 ± 0.7*	63.9 ± 0.3*	70.1 ± 2.5
Years since menopause					14.9 ± 0.4*	14.9 ± 0.7*	14.9 ± 0.3*	20.9 ± 2.7
BMD values measured by pQCT (mg/cm³)								
D50	244.4 ± 3.9	248.1 ± 8.3	245.1 ± 3.5	280.0 ± 40.4	164.0 ± 2.8*	164.6 ± 5.4*	164.1 ± 2.5*	115.6 ± 19.8
D100	605.3 ± 5.5	605.5 ± 11.8	605.3 ± 5.0	618.0 ± 57.1	443.6 ± 4.4*	446.3 ± 8.5*	444.2 ± 3.9†	356.4 ± 31.1
P100	1360.1 ± 8.6	1358.7 ± 18.3	1359.9 ± 7.8	1334.0 ± 88.7	1081.6 ± 7.6*	1078.4 ± 14.7*	1081.0 ± 6.7†	939.5 ± 54.1
BMD values measured by DXA (g/cm²)								
Total body	1.093 ± 0.006	1.093 ± 0.012	1.093 ± 0.005	1.115 ± 0.061	0.923 ± 0.004†	0.911 ± 0.009*	0.920 ± 0.004†	0.817 ± 0.032
L2–L4	1.024 ± 0.008	1.029 ± 0.018	1.024 ± 0.007	1.039 ± 0.088	0.809 ± 0.006	0.815 ± 0.012	0.810 ± 0.005*	0.715 ± 0.044
Femoral neck	0.772 ± 0.007	0.770 ± 0.014	0.772 ± 0.006	0.694 ± 0.070	0.644 ± 0.004*	0.647 ± 0.008*	0.645 ± 0.004‡	0.562 ± 0.030
Trochanter	0.658 ± 0.006	0.658 ± 0.013	0.658 ± 0.005	0.594 ± 0.063	0.543 ± 0.004†	0.535 ± 0.008‡	0.541 ± 0.004§	0.440 ± 0.030
Ward's triangle	0.660 ± 0.009	0.656 ± 0.019	0.659 ± 0.008	0.593 ± 0.092	0.455 ± 0.006‡	0.454 ± 0.011*	0.454 ± 0.005†	0.331 ± 0.042

Data are means ± SE. pQCT, peripheral quantitative computed tomography; DXA, dual-energy X-ray absorptiometry.
*$P < 0.05$.
†$P < 0.005$.
‡$P < 0.01$.
§$P < 0.001$ versus *GG*.

woven bone, osteopenia, fractures, and progressive skeletal deformity, in Navajo individuals homozygous for a deletion of ~100 kb in *OPG* [30].

Previously described SNPs in *OPG* include 163A → G, 209G → A, 245T → G, 889C → T, 950T → C, 1181G → C, and 6890A → C [13–16]. Of these polymorphisms, we selected 950T → C, which is identical to the −223T → C SNP [13], because it was previously shown to be associated with radial BMD in postmenopausal Japanese women, with the *TT* genotype representing a risk factor for reduced BMD [13]. We chose the 245T → G SNP because it was previously associated with the prevalence of osteoporosis and vertebral fracture [16]. We have now shown that the 950T → C SNP was associated with BMD for the proximal radius in premenopausal Japanese women, with the *CC* genotype representing a risk factor for reduced BMD. The reason for the discrepancy between our results and those of the previous study of this polymorphism [13] is unclear, but there are several differences between the two studies. The skeletal sites and methods of BMD measurement thus differed; whereas BMD for total radial bone, including both cancellous and cortical bone, was measured by DXA in the previous study [13], BMD for the proximal radius, including mostly cortical bone, was measured by pQCT in our study. Age and menopausal state of subjects also differed between the two studies; the subjects were postmenopausal women (mean age, 73.2 years) in the previous study [13], whereas the subjects were premenopausal women (mean age, 46.2 years) in our study. Given that the 950T → C SNP is located in the promoter region of *OPG* and was associated with BMD in premenopausal, but not postmenopausal, women in our study, we hypothesize that this polymorphism might affect the promoter activity of *OPG* and that this effect may be estrogen-dependent.

Both 209G → A and 245T → G SNPs of the *OPG* promoter have previously been associated with BMD for the lumbar spine in postmenopausal Slovenian women, with the *209GA/245TG* genotype representing a risk factor for reduced BMD [14]. The 163A → G and 245T → G SNPs were also associated with vertebral fractures in Danish women and men, with the *G* allele of each SNP representing a risk factor for fracture [16]. We have now shown that the 245T → G SNP was associated with BMD for the radius, total body, femoral neck, trochanter, and Ward's triangle in postmenopausal Japanese women, with the *GG* genotype representing a risk factor for reduced BMD, consistent with these previous observations [14,16]. However, the distribution of 245T → G genotypes was significantly different between the studies of Arko et al. [14] (*TT*, 89.3%; *TG*, 10.7%; *GG*, 0%; $P = 0.0328$, χ^2 test) or Langdahl et al. [16] (*TT*, 90.3%; *TG*, 9.2%; *GG*, 0.5%; $P < 0.0001$) and our study (women: *TT*, 78.8%; *TG*, 19.9%; *GG*, 1.3%). These differences in genotype distribution may be attributable to ethnic differences.

The molecular mechanisms that underlie the association of the 950T → C or 245T → G SNPs of *OPG* with BMD remain unclear. The effects of these SNPs on the transcriptional activity of the *OPG* promoter have not been determined. It is possible that the SNPs examined in our study are in linkage disequilibrium with polymorphisms of other nearby genes that are actually responsible for the association with BMD.

In conclusion, the 245T → G SNP of *OPG* was associated with BMD at various sites in postmenopausal Japanese women in the present study, consistent with its previously demonstrated associations with the prevalence of osteoporosis and vertebral fracture in Danish women [16] and with BMD for the lumbar spine in postmenopausal Slovenian women [14]. The 950T → C SNP was also associated with BMD for the proximal radius in premenopausal Japanese women in the present study, although the effect of this SNP on BMD was relatively small. Our present results thus suggest that *OPG* is a candidate locus for reduced bone mass in women.

References

[1] J.A. Kanis, L.J. Melton III, C. Christiansen, C.C. Johnston, N. Khaltaev, The diagnosis of osteoporosis, J. Bone Miner. Res. 9 (1994) 1137–1141.

[2] N.A. Pocock, J.A. Eisman, J.L. Hopper, M.G. Yeates, P.N. Sambrook, S. Eberl, Genetic determinations of bone mass in adults: a twin study, J. Clin. Invest. 80 (1987) 706–710.

[3] M.L. Johnson, G. Gong, W. Kimberling, S.M. Recker, D.B. Kimmel, R.B. Recker, Linkage of a gene causing high bone mass to human chromosome 11 (11q12-13), Am. J. Hum. Genet. 60 (1997) 1326–1332.

[4] M. Devoto, K. Shimoya, J. Caminis, J. Ott, A. Tenenhouse, M.P. Whyte, L. Sereda, S. Hall, E. Considine, C.J. Williams, G. Tromp, H. Kuivaniemi, L. Ala-Kokko, D.J. Prockop, L.D. Spotila, First-stage autosomal genome screen in extended pedigrees suggests genes predisposing to low bone mineral density on chromosomes 1p, 2p and 4p, Eur. J. Hum. Genet. 6 (1998) 151–157.

[5] N.A. Morrison, J.C. Qi, A. Tokita, P.J. Kelly, L. Crofts, T.V. Nguyen, P.N. Sambrook, J.A. Eisman, Prediction of bone density from vitamin D receptor alleles, Nature 367 (1994) 284–287.

[6] A.G. Uitterlinden, H. Burger, Q. Huang, F. Yue, F.E.A. McGuigan, S.F.A. Grant, A. Hofman, J.P.T.M. van Leeuwen, H.A.P. Pols, S.H. Ralston, Relation of alleles of the collagen type Iα1 gene to bone density and the risk of osteoporotic fractures in postmenopausal women, N. Engl. J. Med. 338 (1998) 1016–1021.

[7] Y. Yamada, F. Ando, N. Niino, H. Shimokata, Transforming growth factor-β1 gene polymorphism and bone mineral density, JAMA 285 (2001) 167–168.

[8] W.S. Simonet, D.L. Lacey, C.R. Dunstan, M. Kelley, M.S. Chang, R. Luthy, H.Q. Nguyen, S. Wooden, L. Bennett, T. Boone, G. Shimamoto, M. DeRose, R. Elliott, A. Colombero, H.L. Tan, G. Trail, J. Sullivan, E. Davy, N. Bucay, L. Renshaw-Gegg, T.M. Hughes, D. Hill, W. Pattison, P. Campbell, W.J. Boyle, Osteoprotegerin: a novel secreted protein involved in the regulation of bone density, Cell 89 (1997) 309–319.

[9] N. Bucay, I. Sarosi, C.R. Dunstan, S. Morony, J. Tarpley, C. Capparelli, S. Scully, H.L. Tan, W. Xu, D.L. Lacey, W.J. Boyle, W.S. Simonet, Osteoprotegerin-deficient mice develop early onset

osteoporosis and arterial calcification, Genes Dev. 12 (1998) 1260–1268.

[10] H. Yasuda, N. Shima, N. Nakagawa, S.I. Mochizuki, K. Yano, N. Fujise, Y. Sato, M. Goto, K. Yamaguchi, M. Kuriyama, T. Kanno, A. Murakami, E. Tsuda, T. Morinaga, K. Higashio, Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro, Endocrinology 139 (1998) 1329–1337.

[11] P.J. Bekker, D. Holloway, A. Nakanishi, M. Arrighi, P.T. Leese, C.R. Dunstan, The effect of a single dose of osteoprotegerin in postmenopausal women, J. Bone Miner. Res. 16 (2001) 348–360.

[12] J.J. Body, P. Greipp, R.E. Coleman, T. Facon, F. Geurs, J.P. Fermand, J.L. Harousseau, A. Lipton, X. Mariette, C.D. Williams, A. Nakanishi, D. Holloway, S.W. Martin, C.R. Dunstan, P.J. Bekker, A phase I study of AMGN-0007, a recombinant osteoprotegerin construct, in patients with multiple myeloma or breast carcinoma related bone metastases, Cancer 97 (2003) 887–892.

[13] H. Ohmori, Y. Makita, M. Funamizu, K. Hirooka, T. Hosoi, H. Orimo, T. Suzuki, K. Ikari, T. Nakajima, I. Inoue, A. Hata, Linkage and association analyses of the osteoprotegerin gene locus with human osteoporosis, J. Hum. Genet. 47 (2002) 400–406.

[14] B. Arko, J. Prezelj, R. Komel, A. Kocijancic, P. Hudler, J. Marc, Sequence variations in the osteoprotegerin gene promoter in patients with postmenopausal osteoporosis, J. Clin. Endocrinol. Metab. 87 (2002) 4080–4084.

[15] F. Wynne, F. Drummond, K. O'Sullivan, M. Daly, F. Shanahan, M.G. Molloy, K.A. Quane, Investigation of the genetic influence of the OPG, VDR (FokI), and COLIA1 SpI polymorphisms on BMD in the Irish population, Calcif. Tissue Int. 71 (2002) 26–35.

[16] B.L. Langdahl, M. Carstens, L. Stenkjaer, E.F. Eriksen, Polymorphisms in the osteoprotegerin gene are associated with osteoporotic fractures, J. Bone Miner. Res. 17 (2002) 1245–1255.

[17] H. Shimokata, F. Ando, N. Niino, A new comprehensive study on aging—the National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS–LSA), J. Epidemiol. 10 (2000) S1–S9.

[18] Y. Yamada, F. Ando, N. Niino, H. Shimokata, Association of a polymorphism of the matrix metalloproteinase-1 gene with bone mineral density, Matrix Biol. 21 (2002) 389–392.

[19] Y. Yamada, H. Izawa, S. Ichihara, F. Takatsu, H. Ishihara, H. Hirayama, T. Sone, M. Tanaka, M. Yokota, Prediction of the risk of myocardial infarction from polymorphisms in candidate genes, N. Engl. J. Med. 347 (2002) 1916–1923.

[20] D.M. Anderson, E. Maraskovsky, W.L. Billingsley, W.C. Dougall, M.E. Tometsko, E.R. Roux, M.C. Teepe, R.F. DuBose, D. Cosman, L. Galibert, A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function, Nature 390 (1997) 175–179.

[21] H. Hsu, D.L. Lacey, C.R. Dunstan, I. Solovyev, A. Colombero, E. Timms, H.L. Tan, G. Elliott, M.J. Kelley, I. Sarosi, L. Wang, X.Z. Xia, R. Elliott, L. Chiu, T. Black, S. Scully, C. Capparelli, S. Morony, G. Shimamoto, M.B. Bass, W.J. Boyle, Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand, Proc. Natl. Acad. Sci. USA 96 (1999) 3540–3545.

[22] D.L. Lacey, E. Timms, H.L. Tan, M.J. Kelley, C.R. Dunstan, T. Burgess, R. Elliott, A. Colombero, G. Elliott, S. Scully, H. Hsu, J. Sullivan, N. Hawkins, E. Davy, C. Capparelli, A. Eli, Y.X. Qian, S. Kaufman, I. Sarosi, V. Shalhoub, G. Senaldi, J. Guo, J. Delaney, W.J. Boyle, Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation, Cell 93 (1998) 165–176.

[23] H. Yasuda, N. Shima, N. Nakagawa, K. Yamaguchi, M. Kinosaki, S. Mochizuki, A. Tomoyasu, K. Yano, M. Goto, A. Murakami, E. Tsuda, T. Morinaga, K. Higashio, N. Udagawa, N. Takahashi, T. Suda, Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL, Proc. Natl. Acad. Sci. USA 95 (1998) 3597–3602.

[24] K. Fuller, B. Wong, S. Fox, Y. Choi, T.J. Chambers, TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts, J. Exp. Med. 188 (1998) 997–1001.

[25] T.L. Burgess, Y. Qian, S. Kaufman, B.D. Ring, G. Van, C. Capparelli, M. Kelley, H. Hsu, W.J. Boyle, C.R. Dunstan, S. Hu, D.L. Lacey, The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts, J. Cell. Biol. 145 (1999) 527–538.

[26] M. Kanematsu, T. Sato, H. Takai, K. Watanabe, K. Ikeda, Y. Yamada, Prostaglandin E2 induces expression of receptor activator of nuclear factor-kappa B ligand/osteoprotegerin ligand on pre-B cells: implications for accelerated osteoclastogenesis in estrogen deficiency, J. Bone Miner. Res. 15 (2000) 1321–1329.

[27] H. Takai, M. Kanematsu, K. Yano, E. Tsuda, K. Higashio, K. Ikeda, K. Watanabe, Y. Yamada, Transforming growth factor-β stimulates the production of osteoprotegerin/osteoclastogenesis inhibitory factor by bone marrow stromal cells, J. Biol. Chem. 273 (1998) 27091–27096.

[28] Y. Hakeda, Y. Kobayashi, K. Yamaguchi, H. Yasuda, E. Tsuda, K. Higashio, T. Miyata, M. Kumegawa, Osteoclastogenesis inhibitory factor (OCIF) directly inhibits bone-resorbing activity of isolated mature osteoclasts, Biochem. Biophys. Res. Commun. 251 (1998) 796–801.

[29] T. Akatsu, T. Murakami, M. Nishikawa, K. Ono, N. Shinomiya, E. Tsuda, S. Mochizuki, K. Yamaguchi, M. Kinosaki, K. Higashio, M. Yamamoto, K. Motoyoshi, N. Nagata, Osteoclastogenesis inhibitory factor suppresses osteoclast survival by interfering in the interaction of stromal cells with osteoclasts, Biochem. Biophys. Res. Commun. 250 (1998) 229–234.

[30] M.P. Whyte, S.E. Obrecht, P.M. Finnegan, J.L. Jones, M.N. Podgornik, W.H. McAlister, S. Mumm, Osteoprotegerin deficiency and juvenile Paget's disease, N. Engl. J. Med. 347 (2002) 175–184.